University of Alberta

Hormonal Regulation in Early Pea Fruit Development

By

Phuong Ngo

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master in Science

Department of Agricultural, Food and Nutritional Sciences

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Oct 25, 1999

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Abstract

Normal pea pericarp growth requires the presence of seeds. Removal of seeds results in reduced pericarp growth and subsequent abscission. It is proposed that pea seeds may promote pericarp growth by maintaining gibberellin (GA) biosynthesis in the pericarp through seed transmittable factors such as auxin. This research focused on the use of 4-substituted auxins as molecular tools to determine the specificity of auxin regulation of GA 20-oxidase expression and enzyme activity in pea pericarp. Through growth studies, northern blot analysis and [^{14}C]GA_{19} metabolism profiles, we have gathered results that demonstrate that GA 20-oxidase gene expression and enzyme activity are specific to the biologically active auxins and that 4-Cl-IAA regulation of GA 20-oxidase gene expression is dose dependent. Application of the naturally occurring auxin 4-Cl-IAA to deseeded pea pericarp resulted in stimulated growth, GA 20-oxidase mRNA expression and increased [^{14}C]GA_{19} metabolism to [^{14}C]GA_{20} over all other 4-substituted auxins tested including IAA. These data support the hypothesis that 4-Cl-IAA regulates synthesis of active GAs in the pericarp and plays an important role in the regulation of early pea fruit development.

For my family, for their everlasting support and encouragement.

(Cho Gia Đình của tôi: Anh, Má, Anh Xiu, Anh Dòn va Bé)

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List of Abbreviations

ANOVA	analysis of variance
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CDP	copalyldiphosphate
CTP	cytosine triphosphate
DAA	days after anthesis
DNA	deoxyribonucleic acid
EtBR	ethidium bromide
4-Cl-IAA	4-chloroindole-3-acetic-acid
4-Et-IAA	4-ethylindole-3-acetic acid
4-F-IAA	4-fluoroindole-3-acetic acid
4-Me-IAA	4-methylindole-3-acetic acid
GA	gibberellin
GC-MS-SIM	gas chromatography-mass spectrometry-selective ion monitoring
GGPP	geranyl geranyl pyrophosphate
NAA	naphthaleneacetic acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
kDa	kilodalton
KSA	*ent*-kaurene synthase A
KSB	*ent*-kaurene synthase B

MOPS	3-N-morpholino-propane sulfonic acid
mRNA	messenger ribonucleic acid
MVA	mevalonic acid
RT-PCR	reverse transcriptase polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SDS	lauryl sulfate sodium salt
SE	standard error
SP	split pod
SPNS	split pod no seeds
SSC	sodium chloride/sodium citrate
SSPE	sodium chloride/sodium phosphate
	ethylene diamine tetra acetic acid
STS	silver thiosulfate
WT	wild type

Chapter 1

Introduction

1.1 Fruit Development

Fruit growth involves an interaction of complex regulatory mechanisms that control the division, growth and differentiation of plant cells (Gillaspy et al., 1993). The development of fruits involves three developmental phases, each of which is dependent on the earlier phase.

The first phase of fruit growth involves ovary development, fertilization and fruit set. This phase is dependent upon the successful completion of pollination and fertilization, which determines whether ovary development will occur. Completion of this process is known as fruit set (Gillaspy et al., 1993). Natural plant hormones such as auxins and gibberellins (GAs) produced by pollen are hypothesized to play an important role in signaling subsequent activation of cell division for further fruit maturation (Gillaspy et al., 1993). Application of GAs and auxin to ovaries can result in fruit set in the absence of fertilization (tomato: Gustafson, 1960; Nitsch, 1960; pea: Garcia-Martinez and Carbonell, 1980). As well as in tobacco, substaintable auxin levels are detected after pollination has occurred (Muir, 1942).

The second phase of fruit development involves pericarp cellular division and elongation, seed formation and early embryo development (Tomato: Varga and Bruinsma, 1986; Pea: Vercher et al., 1984; Cooper, 1938; Eeuwens and Schwabe, 1975). The presence of fertilized ovules triggers the continued development of the ovary. In apple, ovules that do not develop seeds in part of a fruit result in lopsided fruit formation in which normal and retarded organ development is closely related to the presence or absence of seeds (Roberts, 1946; Nitsch et al., 1960). In *Pisum sativum* a positive correlation between the number of seeds and the ability of a plant to maintain fruit growth was found, thus suggesting that seeds play a vital role in the overall development of the fruit (Ozga et al., 1992). Hormonal signals (GAs and auxins)

originating from the fertilized ovules may be responsible for continued fruit development perhaps by maintaining hormone levels in surrounding tissue (Eeuwens and Schwabe, 1975; Sponsel, 1982).

The final phase of fruit development is cellular expansion of the ovary and embryo maturation (Gillaspy et al., 1993). It is generally accepted that auxins and GAs are responsible for cellular expansion in ovary tissues (Rayle and Cleland, 1992). However, their exact roles are not known (Gillaspy et al., 1993).

Each phase of fruit development requires signal molecules to be produced and to direct cellular activities in the surrounding tissues. We propose to use the pea fruit as a model system to study signal molecules produced by developing fertilized ovules (seeds) that are likely involved in coordinating growth of the surrounding fruit (pericarp) tissue (phase II of fruit growth). The pea is a self pollinating legume that completes its fertilization of ovules prior to full bloom (anthesis) (Cooper, 1938). The growth of the pea pod (pericarp) is linear between 3 and 7 days after anthesis (DAA), after which, the growth rate decreases from 8-9 days until elongation ceases at 12 DAA (Eeuwens and Schwabe, 1975). Seed development lags behind the development of the pericarp (Eeuwens and Schwabe, 1975). Growth of the pea fruit during the first week after full bloom is mainly due to rapid elongation and enlargement of the pod wall. However, as the pod declines in growth rate, there is an associated increase in growth rate of seeds that is maintained until full maturation of the pea fruit (Eeuwens and Schwabe, 1975).

Normal pea pericarp development is dependent on the presence of fertilized ovules. Killing of fertilized ovules (seeds) by needle pricking at 2 DAA results in inhibition of pericarp growth and subsequent abscission of the pericarp (Eeuwens and Schwabe, 1975). However, growth of pericarps containing killed seeds could be restored by exogenous gibberellin (GA_3) and/or auxin (NAA; Eeuwens and Schwabe, 1975). Therefore, it was suggested that seeds play

an important role in early fruit growth by supplying or maintaining GAs and/or auxins required for pericarp growth (Eeuwens and Schwabe, 1975).

To study the signal molecules involved in early fruit development, a split-pericarp system was used that allowed the pericarp to remain attached to the plant and also allowed for easy manipulation of seeds while maintaining viable seeds and elongating pericarp (Ozga et al., 1992). Previous results obtained using this system revealed that seeds are required to maintain pericarp growth and that GAs as well as the auxin, 4-Cl-IAA, can substitute for the seeds in the stimulation of pericarp growth (Reinecke et al., 1995; Ozga and Reinecke., 1999).

1.2 Auxins

Auxins are one class of plant hormones defined by their biological activity, however, naturally occurring auxins are most likely derived from tryptophan or indole precursors (Davies, 1995). The most common naturally occurring auxin in plants is indole-3-acetic acid (IAA). Indole-3-butyric acid (IBA) and the halogenated auxin 4-chloroindole-3-acetic acid (4-Cl-IAA) have also been isolated from a few species (Bandurski et al., 1995). Although 4-Cl-IAA was initially isolated from pea seeds in the late 1960's (Marumo et al., 1968) the biological role of this endogenous halogenated auxin is not known. Reinecke et al. (1995) found that exogenous 4-Cl-IAA could restore pea pericarp development after deseeding. This suggests that the seeds sustain pericarp growth, at least in part, by supplying 4-Cl-AA to surrounding tissue. The presence of 4-Cl-IAA in pea seeds was confirmed by Katayama et al. (1988), however they were unable to detect 4-Cl-IAA in any other plant organs of pea including the pericarp. Recently, through the use of GC-MS selective ion monitoring in the presence of stable-isotope labeled internal standards, Magnus et al. (1997) detected and quantitated 4-Cl-IAA as well as IAA in pea pericarp. Magnus et al. (1997) found the concentrations of 4-Cl-IAA between 3 and 6 DAA, were higher in the seeds than the pericarp. This suggests that the seeds could be an important

source of this auxin for the developing pea pericarp. IAA was found to be more abundant than 4-Cl-IAA in both seeds and pericarp at 3 and 6 DAA (Magnus et al., 1997).

Reinecke et al. (1995, 1999) investigated the effects of indole halogen position and type of indole on biological activity in pea fruit. In this study, the activities of 4-, 5-, 6- and 7- chloro- and fluoro-substituted IAA's were assessed using the pea split-pericarp assay. Results revealed that the indole-type and position of the halogen on the indole ring dramatically affected auxin activity. The naturally occurring auxin, 4-Cl-IAA was most active while 5-Cl-IAA resulted in only moderate pericarp elongation. However, IAA (an endogenous auxin) along with 6- and 7-chloro, and 4-, 5-, 6- and 7-fluoro-substituted IAA were inactive or inhibitory in the assay. In contrast, in pea stems and wheat coleoptile assays, IAA, 4-, 5-, 6- and 7-Cl-IAA as well as 5-F-IAA were all active, although maximal activity was observed at different concentrations (Hoffman et al., 1952; Katekar and Geissler, 1983). Since 4-Cl-IAA is a naturally occurring auxin that exhibits unique activity in stimulating deseeded pericarp growth in pea, it may function as a seed signal to coordinate pea fruit development.

Further analysis of the biological and structural importance of the chemical substitution at the four position of IAA was carried out by Reinecke et al. (1999). The activities of 4-Cl-, 4-Me-, 4-Et-, and 4-F-substituted IAA were assessed in the pea split-pericarp system. The results of the growth activities were: 4-Cl-IAA $\geq$ 4-Me-IAA > 4-Et-IAA > 4-F-IAA $\geq$ IAA (Reinecke et al., 1999). Effects of substitution at the four position of IAA on the physico-chemical properties of these compounds (lipophilicity, acid-base properties of the indole NH and the molecular volume as determined by x-ray crystallography) were determined. The study concluded that optimum size (molecular volume) of the 4-substituent and its lipophilicity were most likely required for maximal auxin growth promotion in pea. Methyl- and chloro-substituents, which are similar in size, had similar growth promoting activities. The larger ethyl- and smaller fluoro- and hydrogen-substituents at the 4- position resulted in lower or no growth promoting activities.

1.3 Gibberellin Biosynthesis

Gibberellins (GA), another class of plant hormones, are characterized as tetracyclic diterpenoid acids with the *ent*-gibberellane ring structure. GA biosynthesis occurs in both vegetative and reproductive plant tissues and accumulation of GAs to high levels in developing seeds occurs in many species (Graebe, 1987). The GA biosynthesis pathway is composed of three stages. Stage I involves the formation of *ent*-kaurene from mevalonic acid. In stage II *ent*-kaurene undergoes a series of oxidative reactions resulting in GA_{12}-aldehyde formation. In stage III, GA_{12}- aldehyde is converted to C-20 and C-19-GAs.

1.4 Stage I of GA Biosynthesis

Mevalonic acid (MVA) undergoes a series of enzymatic reactions to form geranyl-geranyl pyrophosphate (GGPP). GGPP is then cyclized in a two step reaction forming *ent*-kaurene (Figure 1-1). The enzymes that catalyze this cyclization are *ent*-kaurene synthetase A (KSA) (now called copalyl diphosphatase) and *ent*-kaurene synthase B (now called kaurene synthase). The formation of *ent*-kaurene is considered to be the first committed step towards GA biosynthesis.

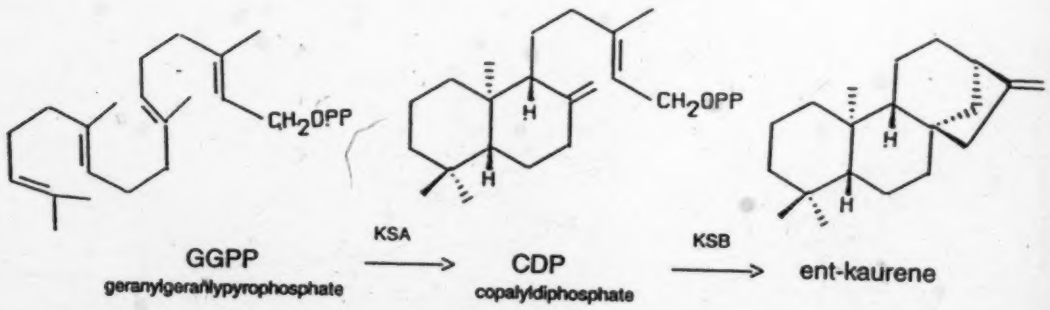

Figure 1-1. (Sponsel, 1995) Stage I of GA biosynthesis.

Cloning and characterization of the GA1 locus in *Arabidopsis*, using the GA-responding male sterile dwarf mutant ga1 and a genomic subtraction technique demonstrated that this gene encodes for the KSA (86 kDa) protein (Sun et al., 1992; Sun and Kamiya, 1994). Sun and Kamiya (1994) were able to introduce a 2.4 kb cDNA clone of GA1 fused to the cauliflower mosaic virus (CaMV) 35S promoter into mutant ga1 plants and obtain successful complementation. Transformed plants exhibited wildtype height and were able to set seeds successfully in the absence of exogenous GAs. Analysis of the first 50 N-terminal amino acids of the KSA protein revealed properties common to that of transit peptides of many chloroplast proteins. In vitro protein import experiments showed that this gene can be imported into pea chloroplasts and processed to a 76 kDa protein (Sun and Kamiya, 1994). Further research on the GA1 locus of *Arabidopsis* by Silverstone et al. (1997) with the use of a GUS reporter gene fusion and RT-PCR have indicated that the *Arabidopsis* KSA-encoding gene is highly regulated during the growth and development of the plant. GUS staining was observed in regions of the plant that were undergoing growth and development, including shoot and root tips, anthers and developing seeds. This data by Silverstone et al. (1997) correlates well with data obtained by Sun and Kamiya (1994) and Aach et al. (1995) that the GA1 promoter appears to be active primarily in cells without mature chloroplasts, i.e. shoot meristem, vascular tissue, root tips and developing seeds.

Similarly, Ait-Ali et al. (1997) cloned the LS locus (codes for the KSA protein in pea) using the dwarf pea ls-1 and showed that the LS fusion proteins of the WT and ls-1 mutants were not the same. The WT fusion protein was able to metabolize the substrates MVA, GGPP and CDP to *ent*-kaurene, whereas the ls-1 fusion protein did not. Ait-Ali et al. (1997) also showed that pea seed KSA is developmentally controlled.

Recently, the gene for KSB has been cloned from pumpkin by Yamaguchi et al. (1996). Results from this study have revealed very high expression of KSB in immature seeds along with

high KSB enzymatic activity. An N-terminal amino acid sequence characteristic of transit peptides was present in the KSB protein. Since Sun and Kamiya (1994) have also presented evidence that KSA is localized to plastids, Yamaguchi et al. (1996) proposed a model in which KSA and KSB form a complex in plastids to efficiently catalyze the conversion of GGPP to *ent*-kaurene.

1.5 Stages II and III of GA Biosynthesis

The latter phases of GA biosynthesis in pea involve the conversion of *ent*-kaurene to GA_{12}-aldehyde (stage II) and the subsequent oxidation of GA_{12}-aldehyde and elimination of carbon 20 (C-20) (stage III) (Figure 1-2. Graebe, 1987). GA_{12}-aldehyde is considered the first committed GA in the GA biosynthesis pathway. GA_{12}-aldehyde is oxidized to GA_{12} and subsequently 13-hydroxylated to GA_{53} (early C-13 hydroxylation pathway). The C-20 carbon of GA_{53} is then oxidized first to an alcohol (GA_{44}) than to an aldehyde (GA_{19}). GA_{19} is converted to GA_{20} by the elimination of C-20, forming a C-19 carbon structure. 3β-hydroxylation of GA_{20} produces GA_1 which is considered to be the biologically active endogenous GA in pea internode elongation (Ingram et al., 1984). It is assumed that GA_1 has a similar function in pea pericarp. 2β-hydroxylation of GA_{20} and GA_1, producing GA_{29} and GA_8, respectively, is considered to result in biological inactivation of these GAs (Sponsel, 1995).

Recently, the gene that codes for the enzyme that is capable of oxidation and elimination of C-20, GA 20-oxidase, has been isolated from pumpkin (*Cucurbita maxima;* Lange et al., 1994), *Arabidopsis* (*Arabidopsis thaliana*; Xu et al., 1995), spinach (*Spinacia oleracea*; Wu et al., 1996), french bean (*Phaseolus vulgaris;* Garcia-Martinez et al., 1997), pea (*Pisum sativum;* Garcia-Martinez et al., 1995; Martin et al., 1996), rice (*Oryza sativa;* Toyomasu et al., 1997), wild cucumber (*Marah macrocarpus;* MacMillan et al., 1997), tobacco (*Nicotiana tabacum;* Kusaba et al., 1998), tomato (*Lycopersicon esculentum*; Rebers et al., 1999) and potato (*Solanum*

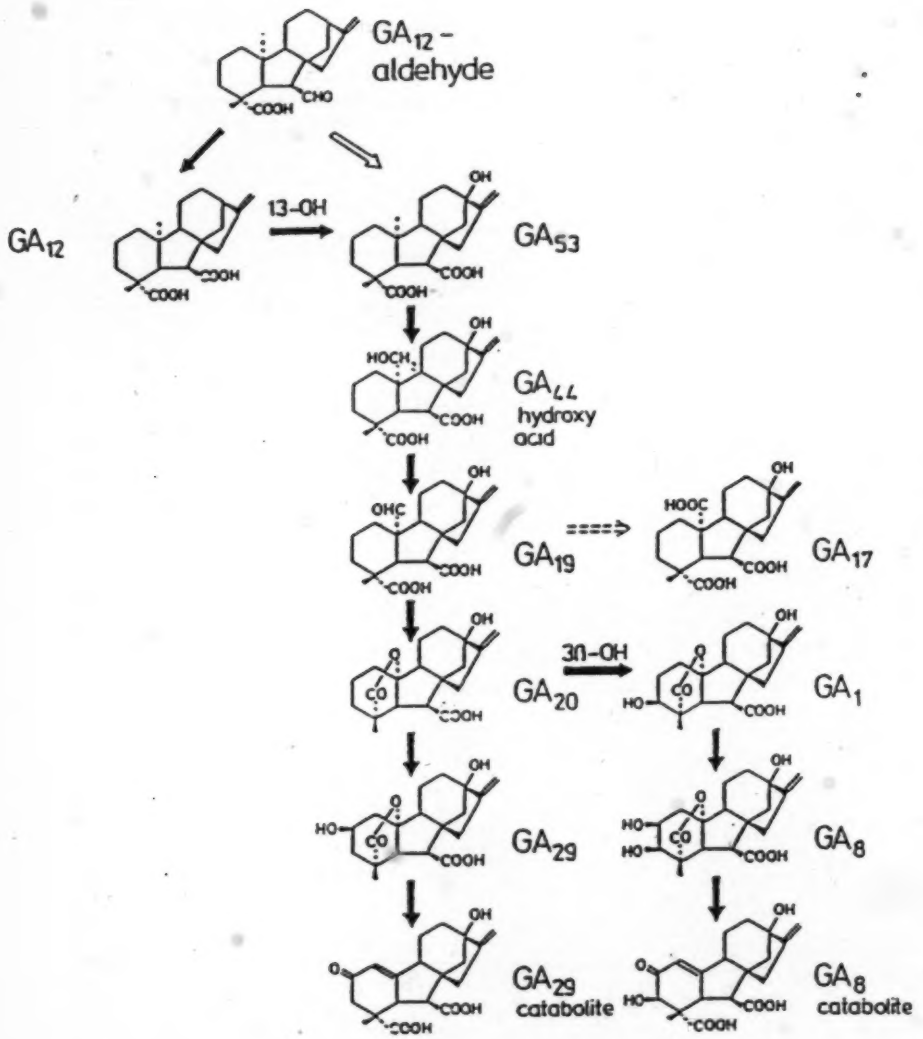

Figure 1-2 (Graebe, 1987). GA biosynthesis pathway in *Pisum sativum*.

tuberosum; Carrera et al., 1999). Heterologous expression of the GA 20-oxidase cDNA's of pumpkin, *Arabidopsis*, spinach, french bean, pea, rice and tomato in *E. coli* has shown that their fusion proteins catalyze the biosynthetic sequence $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$. These data suggest that one enzyme (GA20-oxidase) is responsible for the sequential oxidation and elimination of C-20 of the *ent*-gibberellane ring.

Xu et al. (1995) found the expression of the GA5 locus which encodes for the GA 20-oxidase enzyme in *Arabidopsis* was enhanced under long day conditions. Similarly, in spinach, Wu et al. (1996) also found the expression of GA 20-oxidase to increase under long day conditions, suggesting photoperiodic regulation of GA 20-oxidase expression (Zeevaart et al., 1990). More recent, and similar findings of photoperiodic regulation of GA 20-oxidase have been reported in potato in which a short period of light interruption during the night increased GA 20-oxidase mRNA levels (Carrera et al., 1999).

Martin et al. (1996) observed that transcript levels of GA 20-oxidase decreased upon exogenous GA_3 treatment suggesting feed back regulation of GA 20-oxidase gene expression in pea. Similarly in *Arabidopsis*, Phillips et al. (1995) found that when GA_3 was sprayed to plants, transcript levels of GA 20-oxidase dramatically decreased. Similar recent findings have also been reported in potato (Carrera et al., 1999) and tobacco (Kusaba et al., 1998).

1.6 Interaction of GAs and Auxins

Metabolism studies indicated that pea pericarp has the capacity to metabolize $^{2}H\text{-}GA_{12}$ to $^{2}H\text{-}GA_{53}$ and $^{14}C\text{-}GA_{12}$ to $^{14}C\text{-}GA_{20}$ when seeds are present (Maki and Brenner, 1991; Ozga et al.,1992). However, when seeds were removed, $^{14}C\text{-}GA_{12}$ was metabolized to $^{14}C\text{-}GA_{19}$ but no $^{14}C\text{-}GA_{20}$ was detected (Ozga et al., 1992). Application of 4-Cl-IAA to deseeded pericarp stimulated the conversion of $[^{14}C]\text{-}GA_{19}$ to $[^{14}C]\text{-}GA_{20}$ as well as pericarp growth (van Huizen et

al., 1995). These findings suggested that the biosynthesis of GAs in pea pericarp is influenced by the presence of seeds and that 4-Cl-IAA may be a seed-derived factor that stimulates GA biosynthesis in the pericarp.

Further research in our lab has shown that pericarp GA 20-oxidase mRNA levels are maintained when seeds are present but significantly decrease when seeds are removed from the pericarp (van Huizen et al., 1997). These data demonstrate that seeds are required to maintain pericarp GA 20-oxidase mRNA levels.

When 4-Cl-IAA was applied to deseeded pericarp, GA 20-oxidase mRNA levels significantly increased within 2 hours of application. These results suggest that 4-Cl-IAA can substitute for seeds in maintaining pericarp GA biosynthesis, at least in part by stimulating or maintaining GA 20-oxidase transcript levels. However, experiments using a GA biosynthesis inhibitor, paclobutrazol, demonstrate that 4-Cl-IAA also has a direct auxin effect on pericarp growth (Ozga and Brenner, 1992).

Feedback regulation of GA 20-oxidase expression by GA_3 has been reported in pea stems and in vegetative and floral tissues of *Arabidopsis* (Martin et al., 1996; Phillips et al., 1995). Our lab has found similar evidence when deseeded pea pericarp have been treated with GA_3 (van Huizen et al., 1997). However, the feedback inhibition of GA 20-oxidase expression by GA_3 was delayed by simultaneous application of 4-Cl-IAA (van Huizen et al., 1997). These data demonstrate an interaction of GA_3 and 4-Cl-IAA in the regulation of GA20-oxidase steady-state mRNA levels in this tissue.

One possible seed-regulatory mechanism in young pea fruit would be the export of 4-Cl-IAA from seeds to the pericarp to coordinate growth of the surrounding fruit tissue (pericarp). We hypothesize two regulatory roles for 4-Cl-IAA in controlling pericarp growth: 1) stimulation of GA biosynthesis (conversion of GA_{19} to GA_{20}) at least in part by increasing the level of and/or stability of GA 20-oxidase mRNA, and 2) a direct auxin effect on pericarp growth.

1.7 Objectives

The main objective of my thesis was to test the working hypothesis that endogenous auxin acts as a seed-derived signal to coordinate growth of the surrounding fruit tissue (pericarp). The specific objectives of this study were to:

1) to use the 4-substituted auxins (which were previously determined to possess a range of biological activities in the pea split-pericarp assay system; Reinecke et al., 1999) as molecular tools to determine the specificity of auxin regulation of GA 20-oxidase expression and enzyme activity in pea pericarp.
2) to determine the dose-response relationship of 4-Cl-IAA on GA 20-oxidase expression in pea pericarp.

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Chapter 2

Auxin Specificity of GA 20-oxidase Gene Expression

2.1 Introduction

In pea (*Pisum sativum*), normal pericarp growth requires the presence of seeds (Eeuwens and Schwabe, 1975). Removal or destruction of the seeds 2 to 3 DAA inhibits pericarp growth and usually results in pericarp abscission (Eeuwens and Schwabe, 1975; Ozga et al., 1992). In addition, the number of seeds per pericarp is positively correlated with the ability of the pea pericarp to elongate (Ozga et al., 1992). Hormonal signals originating from the seeds may be responsible for continued fruit development by maintaining hormone levels in the surrounding tissue (Eeuwens and Schwabe, 1975; Sponsel, 1992). Developing pea seeds and pericarp contain GAs (Garcia-Martinez et al., 1991; Rodrigo et al., 1997) and auxins (4-Cl-IAA and IAA; Marumo et al., 1968; Magnus et al., 1997). During the first 6 DAA when the pericarp largely completes its elongation, IAA and 4-Cl-IAA, and their conjugates occur in much higher concentrations in the seeds than in the pericarp (Magnus et al., 1997). This suggests that auxin may be exported from the seeds to the pericarp where it is needed for pericarp growth. Indeed, during early pericarp growth (2 DAA), application of 4-Cl-IAA to deseeded pea pericarp can substitute for seeds and stimulate pericarp growth (Reinecke et al., 1995). However, the other naturally occurring auxin in pea fruit, IAA, inhibits growth in deseeded pericarp (Reinecke et al., 1995). The pea pericarp system therefore responds in a qualitatively different fashion to two auxins which, in a variety of bioassays, showed only quantitative differences in activity (Reinecke, 1999). This suggests a unique way of auxin action based on alternative specific mechanisms of molecular recognition. Initial work comparing the growth promoting properties of 4-, 5-, 6-, and 7-chloro-IAAs and the corresponding fluoro-IAA analogues demonstrated that the substituent at the 4-position of the indole ring was important for biological activity in pea

pericarp growth (Reinecke et al., 1995). To further elucidate the role of auxin in pea pericarp growth, Reinecke et al. (1999) compared the growth-promoting response and the physiochemical properties of 4-Cl-IAA and its 4-substituted analogues: 4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA and IAA. The comparative ability of these auxins to stimulate pea pericarp growth was: 4-Cl-IAA ≥ 4-Me-IAA > 4-Et-IAA > 4-F-IAA ≥ IAA. The 4-substituent's size and its lipophilicity were associated with growth promoting activity on pea pericarp (Reinecke et al., 1999).

Previous studies using the split-pericarp assay have shown that the presence of seeds or the application of 4-Cl-IAA to deseeded pea pericarp stimulated pericarp GA biosynthesis, specifically, the conversion of GA_{19} to GA_{20} (Ozga et al., 1992; van Huizen et al., 1995). The gene that codes for the enzyme responsible for this conversion (elimination of carbon 20 of the *ent*-gibberellin ring; C-20), GA 20-oxidase, has been isolated from a number of species including pumpkin (*Cucurbita maxima*; Lange et al., 1994), *Arabidopsis* (*Arabidopsis thaliana;* Xu et al., 1995), spinach (*Spinacia oleracea*; Wu et al., 1996), french bean (*Phaseolus vulgaris*; Garcia-Martinez et al., 1997), pea (*Pisum sativum*; Garcia-Martinez et al., 1995; Martin et al., 1996), rice (*Oryza sativa*; Toyomasu et al., 1997), wild cucumber (*Marah macrocarpus*; MacMillan et al., 1997), tobacco (*Nicotiana tobacum;* Kusaba et al., 1998), tomato (*Lycopersicon esculentum*; Rebers et al., 1999), and potato (*Solanum tuberosum;* Carrera et al., 1999). Heterologous expression of pumpkin, *Arabidopsis*, spinach, french bean, pea, rice and tomato GA 20-oxidase cDNA's in *E. coli* has shown that their fusion proteins catalyze the biosynthetic sequence $GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$. These data suggests that one enzyme (GA 20-oxidase) is responsible for the sequential oxidation and elimination of C-20.

van Huizen et al. (1997) investigated the expression of GA 20-oxidase during early pea fruit growth. Pericarp GA 20-oxidase mRNA levels were highest from pre-pollination (-2 DAA) through anthesis (0 DAA), then decreased 3-fold by 2 DAA, and remained at these levels through 6 DAA. The early (-2 DAA) high expression of pericarp GA 20-oxidase may indicate that

pericarp-derived GAs are important for ovary development prior to anthesis. The lower but steady levels of pericarp GA 20-oxidase mRNA observed during the phase of rapid pericarp growth (2 to 6 DAA) suggest that maintenance of pericarp GA biosynthesis is important for sustained pericarp growth. GA metabolism studies support that pea pericarp has active GA biosynthesis $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20}$ (Ozga et al., 1992; van Huizen et al., 1995).

Using the split-pericarp assay system, van Huizen et al. (1997) observed that GA 20-oxidase transcript levels in 2 DAA pericarps with seeds remained relatively stable throughout the 36-h treatment period; however, when the seeds were removed the pericarp transcript levels declined. When 2 DAA deseeded pericarps were treated with 4-Cl-IAA, a significant increase in GA 20-oxidase mRNA levels was detected within 2 h and transcript levels remained elevated for up to 12-h after 4-Cl-IAA application. These data suggest that 4-Cl-IAA regulates GA 20-oxidase gene expression in young pea pericarps. Evidence for the regulation of GA 20-oxidase gene expression by photoperiod (Xu et al., 1995; Wu et al., 1996; Carrera et al., 1999) and GA end-product repression (Xu et al., 1995; Phillips et al., 1995; Martin et al., 1996; van Huizen et al., 1997) has also been reported.

To further understand how seeds and auxin regulate GA biosynthesis in pea pericarp, we used 4-substituted auxins that possess a range of biological activities in the split-pericarp assay system (Reinecke et al., 1999) as molecular tools to determine the specificity of auxin regulation of GA 20-oxidase gene expression. Our results show that regulation of GA 20-oxidase expression by auxin is specific, qualitatively and quantitatively, to the biologically active auxins in the split-pericarp assay system and that stimulation of transcript levels by 4-Cl-IAA is dose dependent.

2.2 Methods and Materials

2.2.1 Plant Material and Treatments

Seeds (4 per pot) of *Pisum sativum* L., line I_3 (Alaska type) were germinated in 20 cm pots in a soil mixture of 1:1 Terra Lite 2000 Metro mix: sand (W.R Grace and Co. Ajax, Canada). Plants were grown in a growth chamber (Conviron, Ashville, NC), at 19/17°C (day/night) in a 16 h light/8 h dark photoperiod with cool white fluorescent and incandescent lights, and pots were thinned to 3 plants based on synchronicity of germination. Distance of lights above the plants was maintained at 30 cm with an average photon flux density of 402 $\mu Em^{-2}s^{-1}$. One fruit between the 3rd and 5th flowering nodes was used per plant; subsequent flowers and lateral buds were removed as they developed. Terminal apical meristems of plants were intact and pericarps remained attached to the plant during the entire experiment.

Pericarps were treated with auxins using a split-pod technique developed by Ozga et al. (1992). Pericarps at 2 days after anthesis (DAA) measuring 15 to 20 mm in length were split down the dorsal suture 1 h prior to the 8 h dark period, and seeds were either left intact (SP) or removed (SPNS). Splitting of the pericarp and removal of the seeds were completed 12 h prior to all auxin applications. Pericarps were treated with 4-Cl-IAA, 4-F-IAA, 4-Me-IAA, 4-Et-IAA, or IAA (50 µM in 0.1% (v/v) Tween 80; 30 µL total); additionally 4-Cl-IAA was applied at 1, 10, or 100 µM in 0.1% (v/v) Tween 80 (30 µL total). All solutions were applied directly to the inside surface of the pericarp wall (endocarp). The SP and SPNS controls were treated with 30 µL 0.1% (v/v) Tween 80. Treated pericarps were covered with plastic bags to maintain high humidity. Pericarps were harvested into liquid nitrogen at 4, 8, 12 and 24 h after the hormone treatment and subsequently stored at -80°C until extraction. Seeds, if present, were removed from the pericarp at harvest (Figure 2-1).

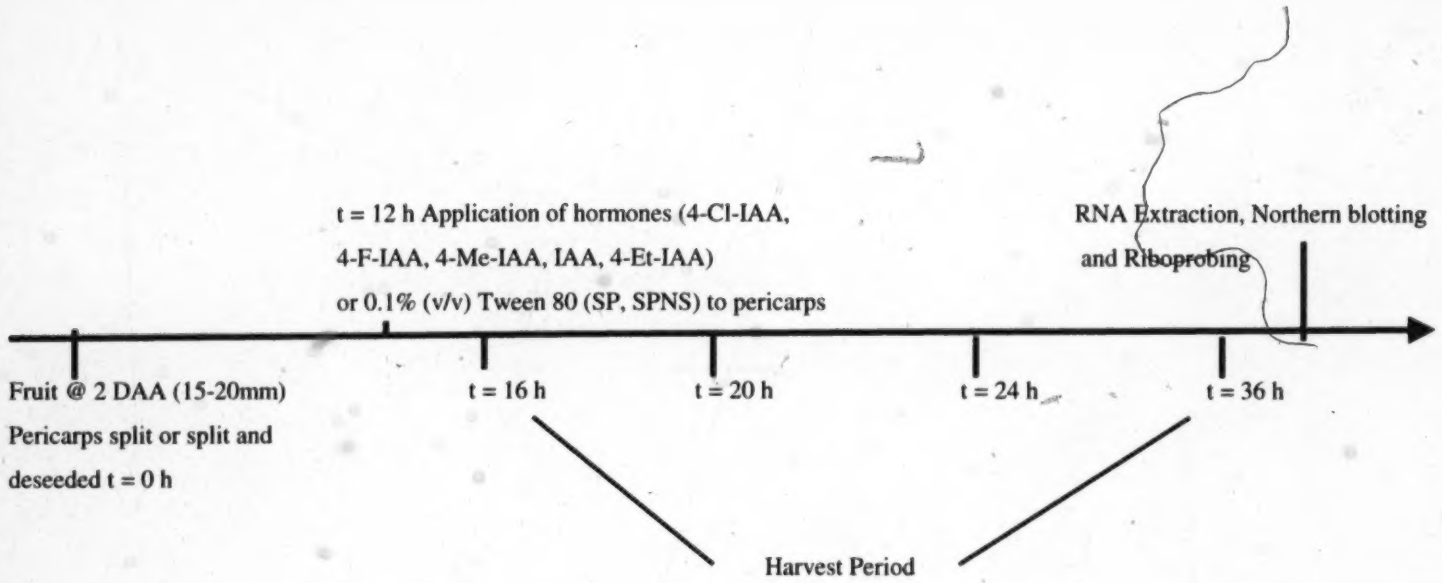

t = 12 h Application of hormones (4-Cl-IAA,
4-F-IAA, 4-Me-IAA, IAA, 4-Et-IAA)
or 0.1% (v/v) Tween 80 (SP, SPNS) to pericarps
RNA Extraction, Northern blotting
and Riboprobing
Fruit @ 2 DAA (15-20mm)
Pericarps split or split and
deseeded t = 0 h
t = 16 h
t = 20 h
t = 24 h
t = 36 h
Harvest Period

Figure 2-1. Time course of hormone application and harvest of pea pericarps.

2.2.2 RNA Isolation and Northern Blot Analysis

Three pods per sample were ground to a fine powder in liquid N_2 (0.3 – 0.5 g) for RNA extraction. Total RNA was extracted following the TriZol (Gibco BRL) procedure based on Chomczynski and Sacchi (1987), with two additional chloroform extractions after the first chloroform extraction to remove polysaccharides. In some samples, 3M LiCl precipitation of RNA was carried out after the second overnight precipitation step instead of two additional chloroform extractions.

For northern blot analysis, total RNA (30 µg per sample) was denatured in 2.2 M formaldehyde/48% formamide and fractionated on a 1.2% (w/v) agarose/2.2 M formaldehyde gel using a 20 mM MOPS buffer (pH 7.0; Maniatis et al., 1982) at a constant 100 volts for 4-5 h. Gels were washed 2 times for 20 minutes in fresh 10X SSC and transferred onto Nylon membranes (Zeta-Probe GT, Bio-Rad) with 10X SSC. RNA integrity was ascertained by ethidium bromide staining of rRNA bands prior to membrane transfer and to confirm uniform transfer of RNA to membranes. RNA was fixed to membranes by baking at 60°C for 2 h under vacuum, and subsequently the membranes were sealed in plastic bags, and stored at 4°C until probing.

A 692 bp sequence of a pea GA 20-oxidase cDNA sequence was used for synthesis of a riboprobe (van Huizen et al., 1997). Briefly, the 692 bp sequence had been ligated into pCR-Script SK (+) (Stratagene) and transformed into *Escherichia coli* strain XL1 Blue. Plasmid preparation of the GA 20-oxidase-containing plasmid was carried out using a Plasmid Pure DNA Miniprep Kit (Sigma). Isolated plasmid was linearized using a *Sma I* restriction enzyme digested at 37°C for 2 h. Subsequently, the enzyme was inactivated by heating for 15 minutes at 65°C. A 1:1:2 phenol:chloroform:ethanol precipitation step was carried out to further purify the digested plasmid. The final isolated GA 20-oxidase linearized plasmid was pooled to a final concentration of 1 µg/µL.

Riboprobe radiolabeling with [^{32}P]CTP was carried out according to the supplier's instructions (Riboprobe *in vitro* Transcription Systems, Promega). Generating an antisense strand to the sequence of interest required a transcription reaction using T3 RNA polymerase and the linearized GA 20-oxidase sequence as a template. Removal of the DNA template was achieved by adding 1 µl RQ1 DNase (1 unit/µL) and 0.5 µl RNasin (36 units/µL) and incubating at 37°C for 15 minutes. Four µl of 5M ammonium acetate and 40 µl of 100% ethanol were added to the mixture which was left to precipitate overnight at -20°C.

Pre-hybridization and hybridization of blots were performed at 50°C in a pre-warmed solution of 60% (v/v) formamide, 1X SSPE, 0.5% (w/v) blotto (low-fat milk powder), 10% (w/v) dextran sulfate, 1% (w/v) SDS, and 0.5 mg/ml denatured salmon sperm DNA (Pharmacia). Blots were pre-hybridized for 1 h. The hybridization solution was prepared separately by heating the denatured salmon sperm DNA in a tube containing 1 ml of 100% (v/v) deionized formamide with 2 x 10^6 dpm/ml of labeled GA 20-oxidase probe at 70°C for 5 minutes prior to addition to the hybridization solution. Blots were hybridized for 18 h and then briefly rinsed in 2X SSC, then washed in 2X SSC, 0.1% (w/v) SDS while gently shaken for 15 minutes at room temperature. A final wash of 0.2X SSC, 1% (w/v) SDS heated to 70°C for 2 to 5 minutes was used as required to obtain minimal background counts using a Geiger counter. For autoradiography, blots were sealed in plastic bags and exposed to Kodak X-Omat AR film at -70°C.

As a developmental control, blots of one experimental replication were also probed with an *Arabidopsis thaliana* actin riboprobe (clone pATC-4 from Dr. Robert J. Ferl, University of Florida, Gainesville). Blots were stripped in a solution of 0.1X SSC, 0.5% (w/v) SDS at 95°C for 20 minutes and re-exposed on X-Omat AR film for 24 h to confirm effective stripping. A 1.8 kb sequence of the original *A. thaliana* actin sequence subcloned into pBluescript II SK vector [by Dr. Mary Christopher and generously donated by Dr. Allen Good University of Alberta, Edmonton] was used. The actin riboprobe was prepared as described above, using the T7 RNA

polymerase to synthesize the antisense probe (pre-hybridization and hybridization performed at 65°C).

2.2.3 mRNA Quantitation

The amount of labeled antisense RNA hybridizing to the RNA blot was determined by scanning the autoradiogram with an imaging densitometer (Bio-Rad). As a loading control ethidium bromide (EtBr) staining of the 18S rRNA band was also quantitated by imaging densitometry, and these values were used to standardize the GA 20-oxidase mRNA signal as follows:

a) GA 20-oxidase = GA 20-oxidase message signal – GA 20-oxidase Background Signal

b) 18 S rRNA = 18S rRNA EtBr Signal – 18S rRNA EtBr Background Signal

c) Loading Control Standard = 18S rRNA of SPNS 12-h treatment

d) Standardized mRNA Signal of Sample = GA 20-oxidase / (18S rRNA / Loading Control Standard)

e) % Relative mRNA Abundance = Standardized mRNA Signal of Sample / Standardized mRNA signal at 12 h x 100

One extraction of 12 pods per replication was performed for 2 DAA (0 h) and the 12 h controls and these samples were run on all gels of the corresponding replication. The value for the GA 20-oxidase signal at the time of hormone application (12 h) on each autoradiogram was designated as 100% and all other signals were calculated relative to that sample to normalize for message recoveries between gel blots.

2.2.4 Statistical Analysis

Growth data (from hormone application to harvest) taken from pericarps used for RNA extraction were analyzed using an analysis of variance (ANOVA) test and tested for linear trends and interactions.

2.3 Results

2.3.1 Auxin Stimulated Pericarp Growth

The length of 2 DAA pericarps with seeds (SP) and deseeded pericarps treated with 50 µM auxin (4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA and IAA) or 0.1% (v/v) Tween 80 (SPNS) increased linearly with time over a 24 h treatment period ($P<0.001$; Figure 2-2). The growth per unit time for SP pericarps and deseeded pericarps treated with 4-Cl-IAA was similar, as indicated by lack of interaction between these two treatments ($P>0.1$). 4-Cl-IAA was more active in stimulating pericarp length in deseeded pericarps than 4-Me-IAA, 4-Et-IAA, 4-F-IAA and IAA (linear interaction of 4-Cl-IAA versus other auxins significant at $P<0.01$). Growth of deseeded pericarps (SPNS) was significantly less than pericarp with seeds (SP) and deseeded pericarps treated with 4-Cl-IAA (linear interaction of SPNS versus SP or 4-Cl-IAA significant at $P<0.01$). However, SPNS pericarp growth was not significantly different than growth of deseeded pericarps treated with 4-Me-IAA, 4-Et-IAA, 4-F-IAA or IAA during this time period (linear interaction not significant, $P>0.1$).

Split pericarps of 2 DAA pollinated ovaries continued to grow in length from 2 to 6 DAA when seeds were present (Figure 2-3). When seeds were removed (SPNS), pericarp growth was inhibited and usually the pericarp abscised within 4 days after seed removal. If 2 DAA deseeded pericarps were treated with 4-Cl-IAA, pericarp growth was restored to the level of pericarps with seeds (SP; 72% greater than SPNS, Figure 2-3). Application of 4-Me-IAA, 4-Et-

Figure 2-2. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA, and IAA on pea pericarp growth over 36 h. Two DAA pericarps were split or split and deseeded and 30 µL of 50 µM auxin or 0.1 % Tween 80 (SP and SPNS) was applied 12 h after deseeding. The arrow indicates the time of hormone application. Data are means ± SE, n = 9.

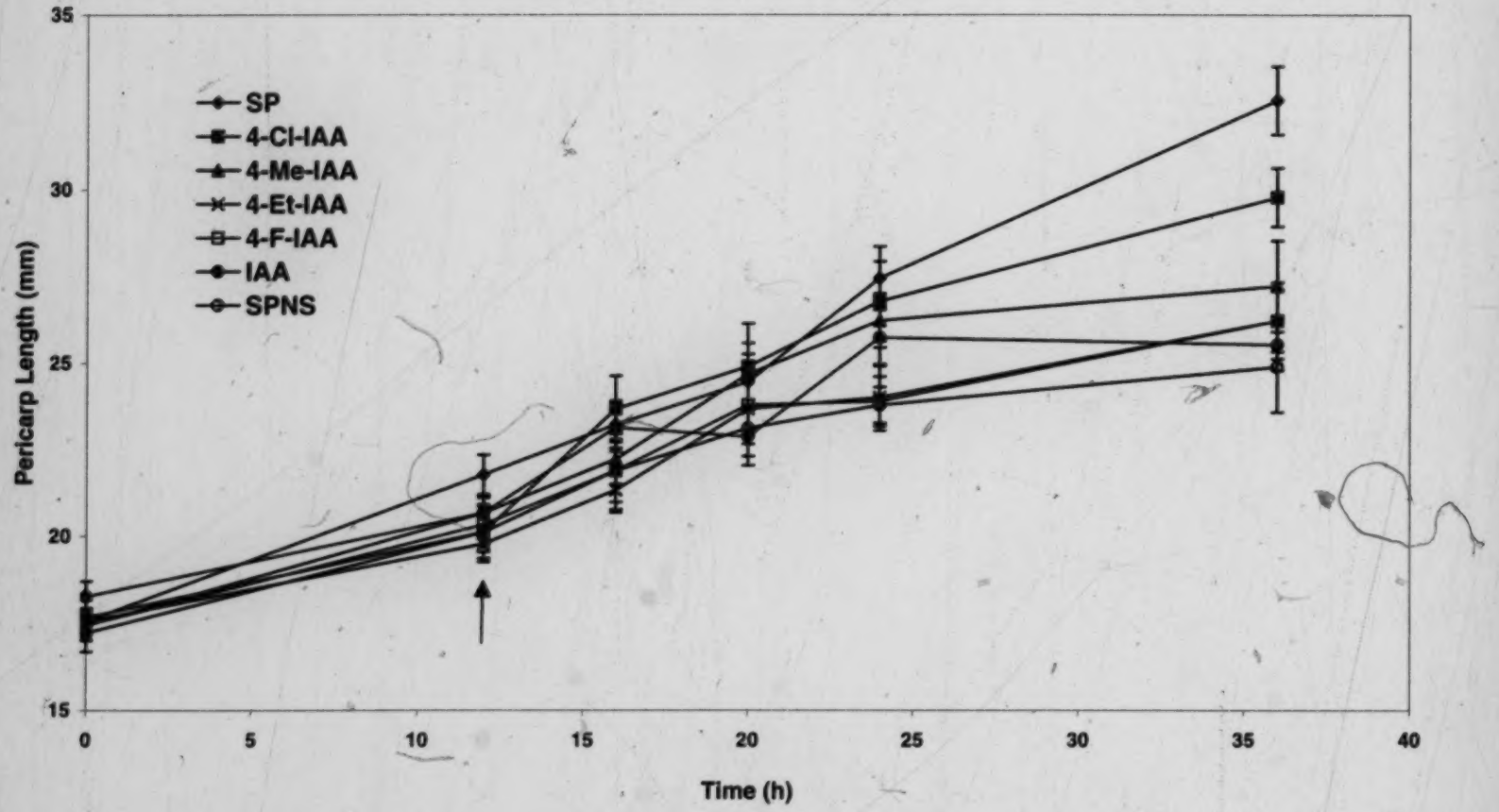
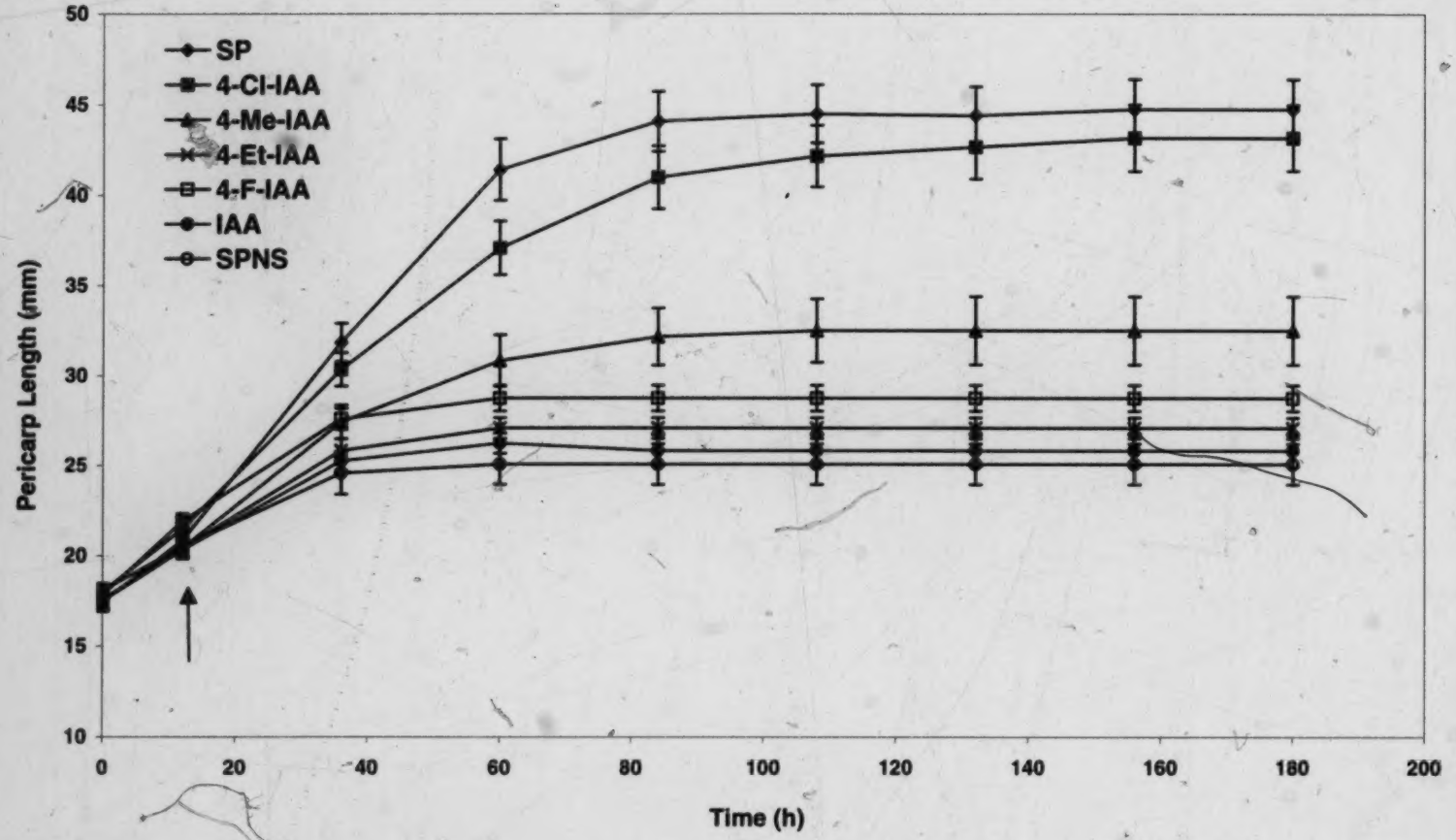

SP
4-Cl-IAA
4-Me-IAA
4-Et-IAA
4-F-IAA
IAA
SPNS
Pericarp Length (mm)
Time (h)
35
30
25
20
15
0
5
10
15
20
25
30
35
40

Figure 2-3. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA and IAA on pea pericarp growth over 7 days. Two DAA pericarps were split or split and deseeded and treated daily for 5 days with 50 µM auxin or 0.1 % Tween 80 (SP and SPNS). The initial hormone treatments were applied 12 h after deseeding. The arrow indicates the time of hormone application. Data are means ± SE, n = 12.


SP
4-Cl-IAA
4-Me-IAA
4-Et-IAA
4-F-IAA
IAA
SPNS
Pericarp Length (mm)
Time (h)
50
45
40
35
30
25
20
15
10
0
20
40
60
80
100
120
140
160
180
200

-IAA and 4-F-IAA to deseeded pericarps stimulated pericarp growth 30%, 8%, and 14% above the SPNS control, respectively, by day 7 of treatment. Application of IAA to deseeded pericarps did not significantly stimulate pericarp growth above the SPNS control (Figure 2-3); by day 7 of treatment these pericarps were flaccid or abscised.

2.3.2 4-Cl-IAA Concentration-Dependent Pericarp Growth

The length of 2 DAA pericarps with seeds (SP) and deseeded pericarps treated with 4-Cl-IAA at 100, 50, 10, 1 or 0 (0.1% (v/v) Tween 80; SPNS) μM increased linearly with time over a 24 h period ($P<0.05$; Figure 2-4). The growth per unit time for SP pericarps and deseeded pericarps treated with 4-Cl-IAA at 100, 50 and 10 μM was similar (linear interaction of SP versus 4-Cl-IAA at 100, 50 and 10 μM not significant; $P>0.1$). Pericarps treated with 1 μM 4-Cl-IAA grew similarly to the SPNS control (linear interaction not significant; $P>0.1$) and significantly less than deseeded pericarps treated with 100, 50 or 10 μM 4-Cl-IAA (linear interaction of 1 μM 4-Cl-IAA versus 100, 50 and 10 μM 4-Cl-IAA significant at $P<0.01$).

Pericarp length (9DAA minus 2DAA) increased linearly with increasing log concentration of 4-Cl-IAA from 1 to 50 μM ($P< 0.001$ Student's T value, $r = 0.846$; Figure 2-6). Application of 100 or 50 μM 4-Cl-IAA to deseeded pericarps stimulated pericarp growth to 55% above the SPNS control 7 days after the initial treatment (Figure 2-5). Growth of deseeded pericarps treated with 50 to 1 μM 4-Cl-IAA decreased with decreasing 4-Cl-IAA concentration when compared to SPNS (10 μM, 34%; 1 μM, 9.2% growth above the SPNS control 7 days after initial treatment).

Figure 2-4. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with 4-Cl-IAA (over a concentration range) on pea pericarp growth over 36 h. Two DAA pericarps were split and deseeded and 30 µL of 100, 50, 10 or 1 µM 4-Cl-IAA or 0.1 % Tween 80 (SP and SPNS) was applied 12 h after deseeding. The arrow indicates the time of hormone application. Data are means ± SE, n=6.

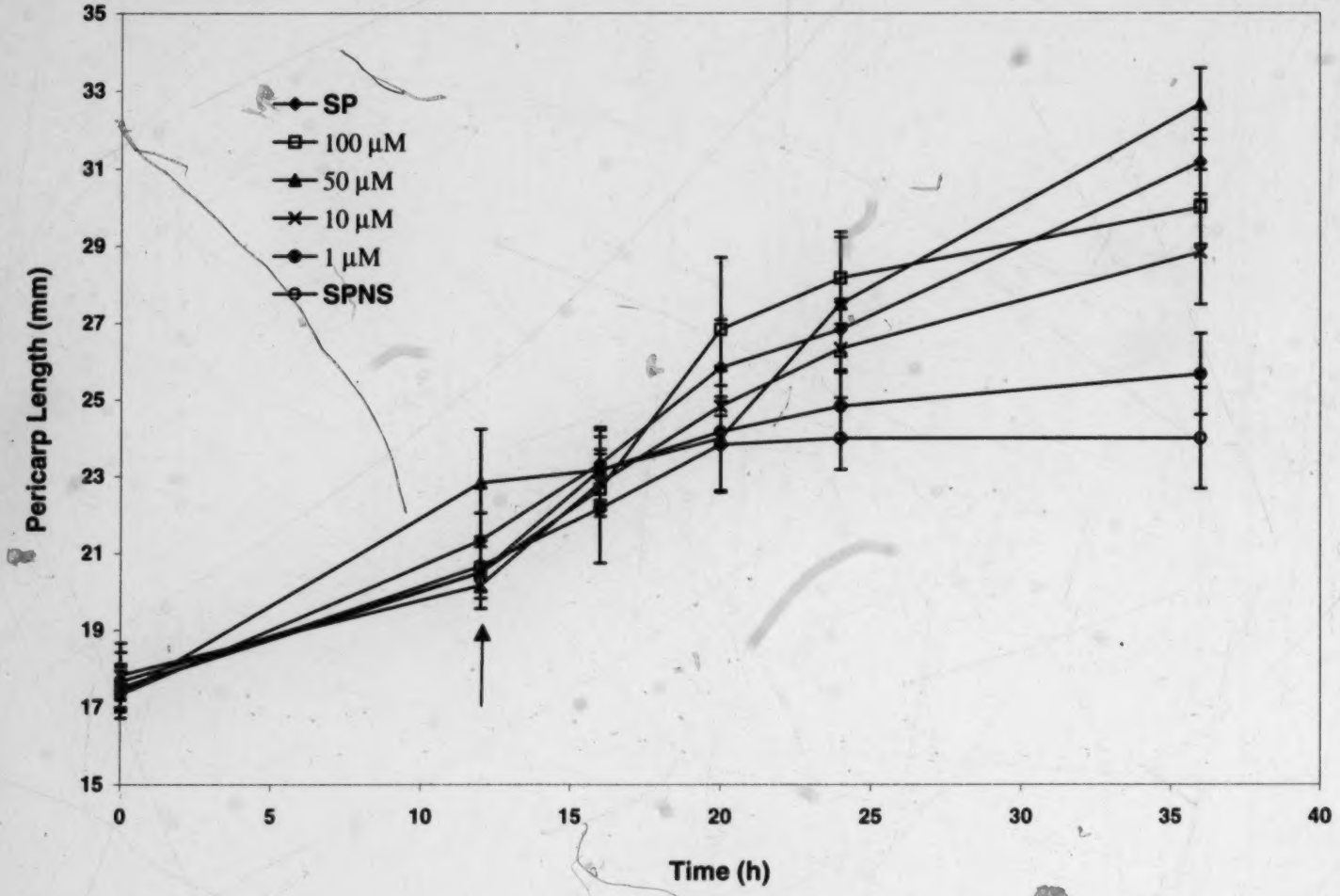

SP
100 µM
50 µM
10 µM
1 µM
SPNS
Pericarp Length (mm)
Time (h)
35
33
31
29
27
25
23
21
19
17
15
0
5
10
15
20
25
30
35
40

Figure 2-5. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with 4-Cl-IAA (over a concentration range) on pea pericarp growth over 7 days. Two DAA pericarps were split and deseeded and treated daily for 5 days with 30 µL of 100, 50, 10 or 1 µM 4-Cl-IAA or 0.1 % Tween 80 (SP and SPNS). The initial hormone treatments were applied 12 h after deseeding. The arrow indicates the time of hormone application. Data are means ± SE, n = 8.

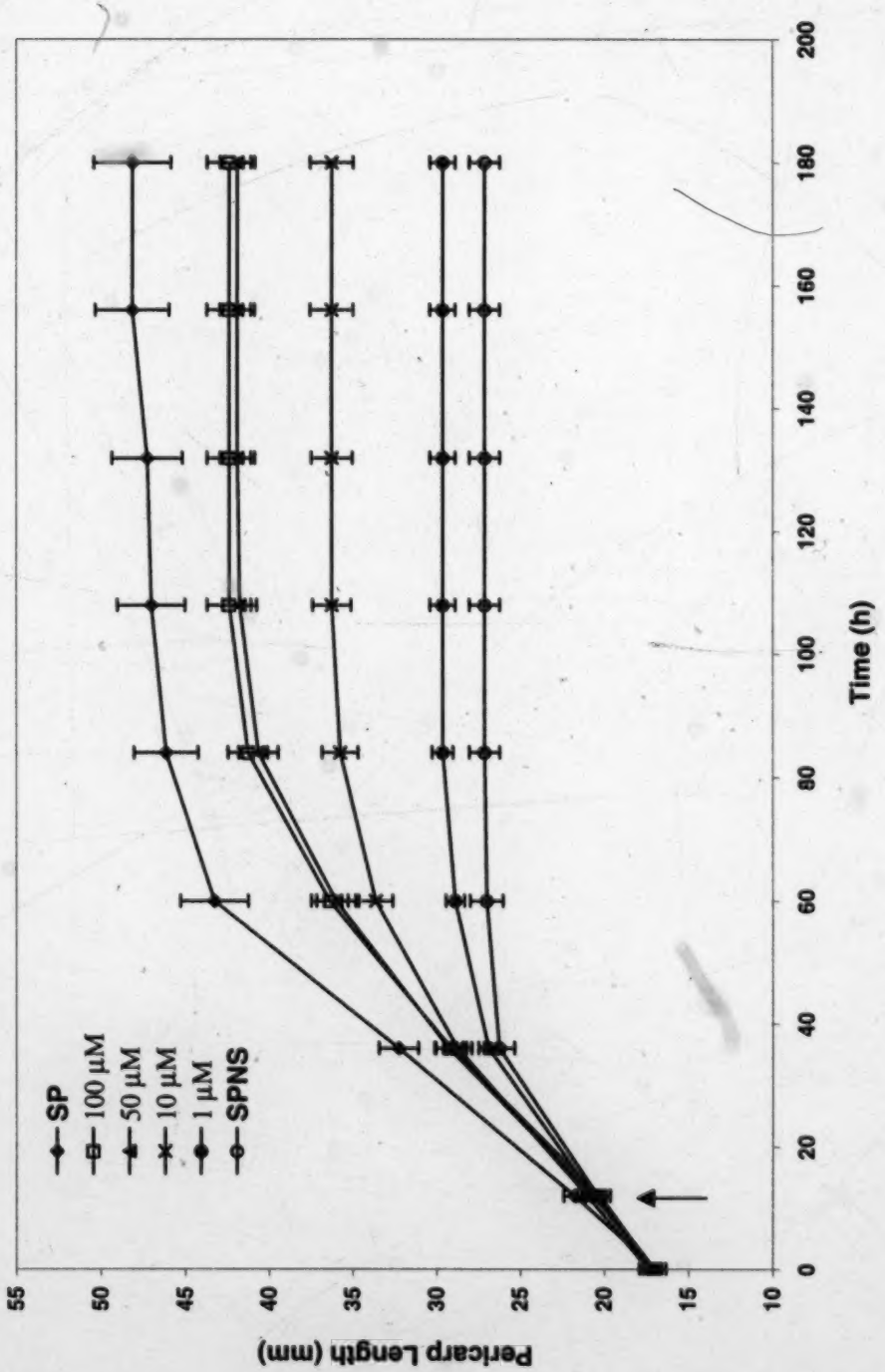
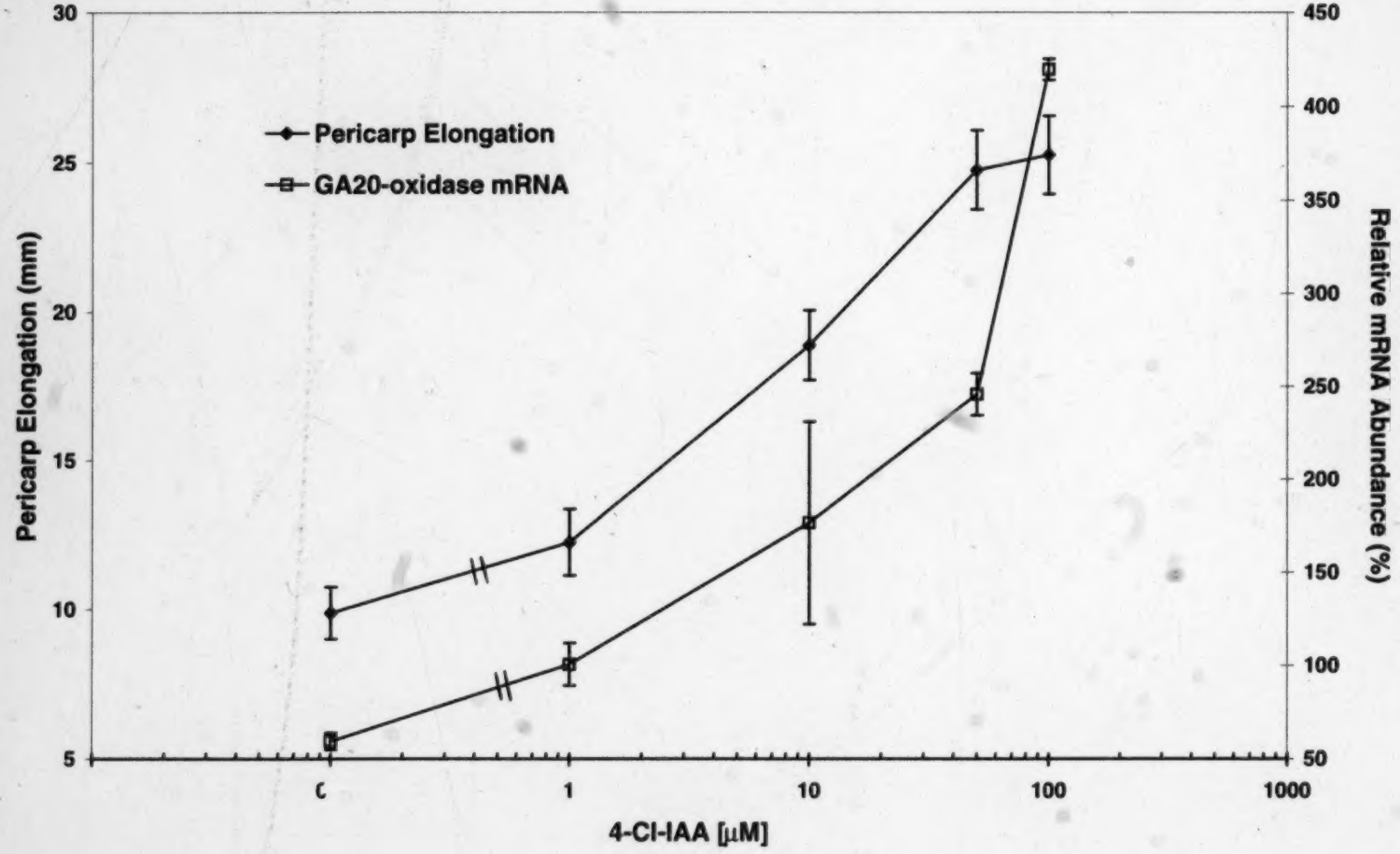

Perlcarp Length (mm)
Time (h)
SP
100 µM
50 µM
10 µM
1 µM
SPNS

Figure 2-6. The effect of increasing auxin concentration on deseeded pericarp elongation (length at 9 DAA minus 2 DAA; m=6.813, b=12.28) and % relative mRNA abundance of GA 20-oxidase (m=137.83, b=73.59). Pericarps were treated as described in Figure 2-4. C = control (0 μM). Data are means ± SE, n = 8.


Pericarp Elongation
GA20-oxidase mRNA
Pericarp Elongation (mm)
Relative mRNA Abundance (%)
4-Cl-IAA [µM]
30
25
20
15
10
5
450
400
350
300
250
200
150
100
50
0
1
10
100
1000

2.3.3 Auxin Stimulation of GA 20-Oxidase Gene Expression

The specificity of auxin stimulation of GA 20-oxidase expression in pea pericarp was investigated using northern blot analysis over a 36 h period (Figures 2-7, 2-8 and 2-9). To allow sufficient time for the pericarp to become depleted of seed-produced factors that might affect pericarp growth, auxins were applied to the pericarps 12 h after deseeding. The average GA 20-oxidase mRNA level in pericarps with seeds (SP), although highly variable among the 3 replications, remained relatively stable during the 36 h period after splitting of the pericarp (Figures 2-7 and 2-8). GA 20-oxidase mRNA levels in deseeded pericarps were similar to levels in the pericarp with seeds during the first 12 h after seed removal; however, after 12 h transcript levels declined reaching a minimum of 36% of the original levels (2 DAA) after 36 h (Figures 2-7 and 2-8).

The highest GA 20-oxidase mRNA levels were observed when deseeded pericarps were treated with the naturally occurring auxin, 4-Cl-IAA (50 µM; Figures 2-7 and 2-9). Transcript levels in the 4-Cl-IAA-treated deseeded pericarps increased significantly within 4 h of hormone application (11.1 times higher than the SPNS control) and remained elevated compared with all other treatments for up to 12 h after the hormone application. 4-Me-IAA was the second most active auxin, significantly increasing GA 20-oxidase mRNA levels above the SPNS control by 5.5 times 4 h after hormone application (Figures 2-7 and 2-9). GA 20-oxidase mRNA levels in 4-Me-IAA-treated deseeded pericarps remained elevated above the 4-Et-IAA-, 4-F-IAA- and IAA-treated deseeded pericarps for 24 h after the hormone application. Application of the 4-substituted IAA analogues, 4-Et-IAA and 4-F-IAA, to deseeded pericarps resulted in small increases in GA 20-oxidase mRNA 8 and 24 h after hormone application. Application of the naturally occurring auxin IAA, to deseeded pericarps resulted in no increase in transcript levels compared to the SPNS control (Figures 2-7 and 2-9).

Figure 2-7. Time course of GA 20-oxidase mRNA accumulation in pea pericarp with seeds (SP), without seeds (SPNS), and pericarp without seeds treated with 4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA, or IAA. Two DAA pericarps were split or split and deseeded and 30 μL of 50 μM auxin or 0.1 % Tween 80 (SP and SPNS) was applied 12 h after deseeding. rRNA banding in a representative gel (SP treatment) is shown prior to membrane transfer (visualized by staining with ethidium bromide). The 18S rRNA band was used as a loading control for all samples and an actin probe was used as a developmental control for one replication of all treatments.

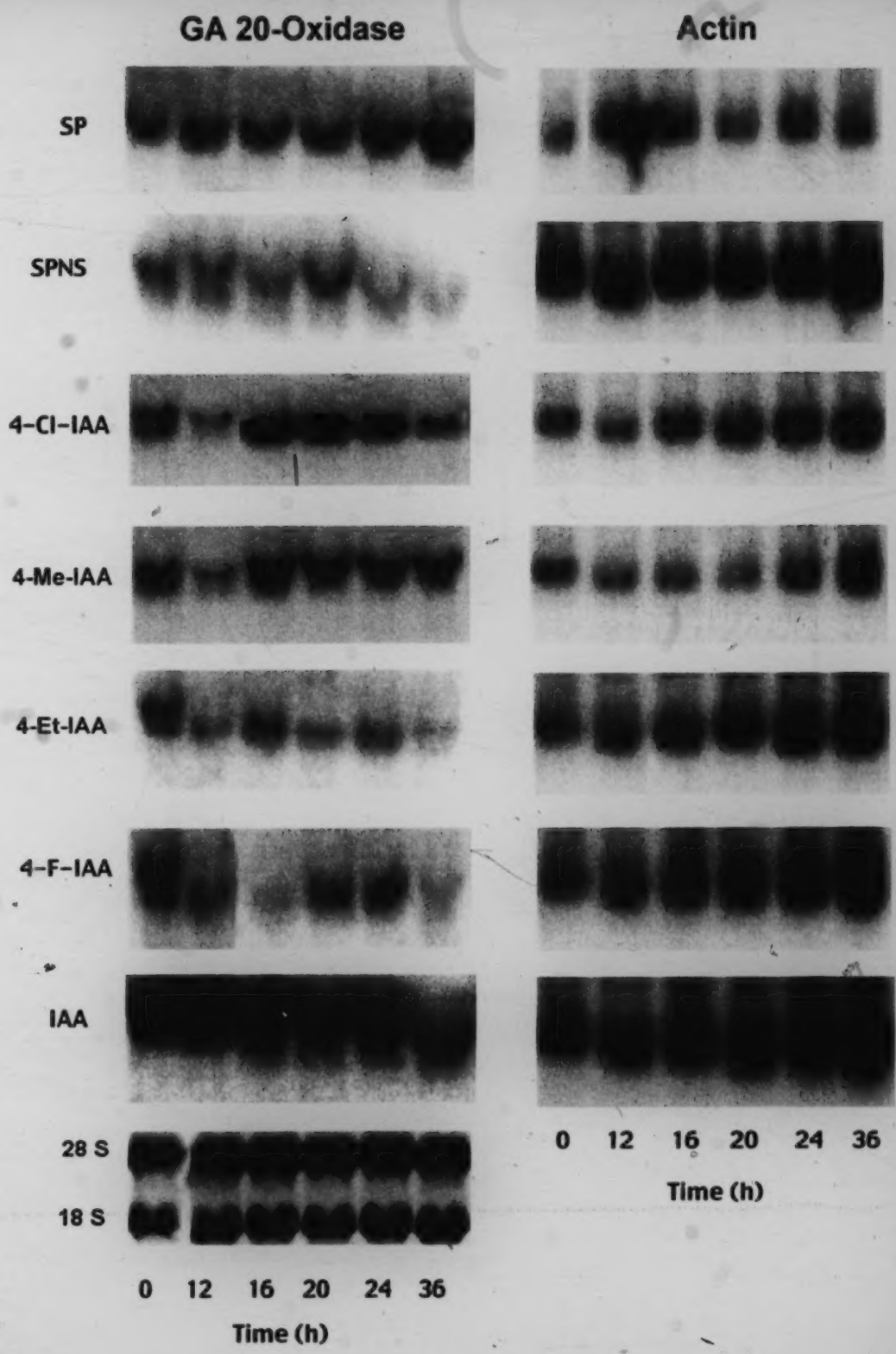

GA 20-Oxidase
Actin
SP
SPNS
4-Cl-IAA
4-Me-IAA
4-Et-IAA
4-F-IAA
IAA
28 S
18 S
0 12 16 20 24 36
Time (h)
0 12 16 20 24 36
Time (h)

Figure 2-8. Relative mRNA abundance of GA 20-oxidase transcripts in pea pericarps with (SP) and without seeds (SPNS) as described in Figure 2-7. Autoradiograms were scanned with an imaging densitometer and these values were normalized to the value for pericarps 12 h after deseeding. Data are means ± SE, n = 3.

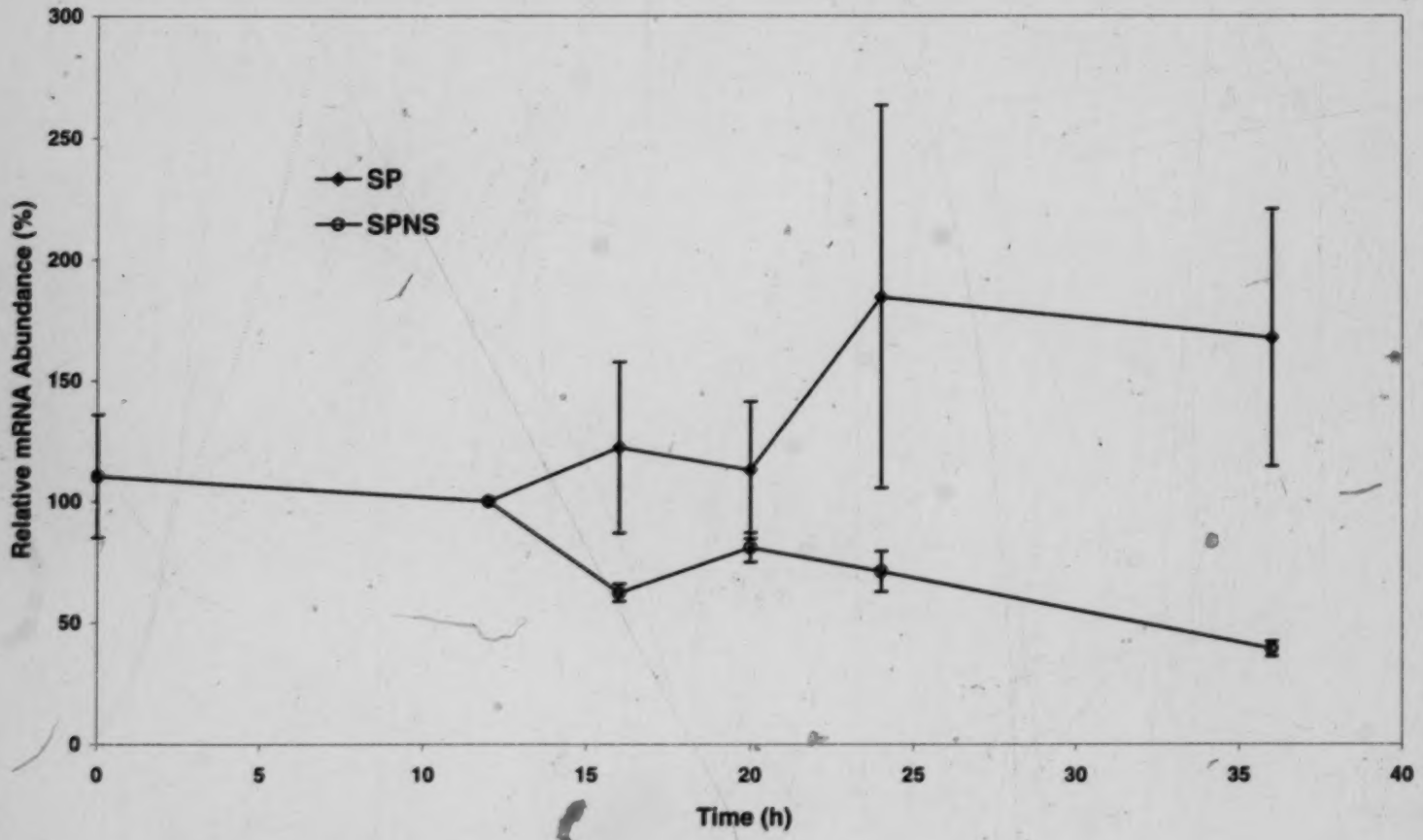
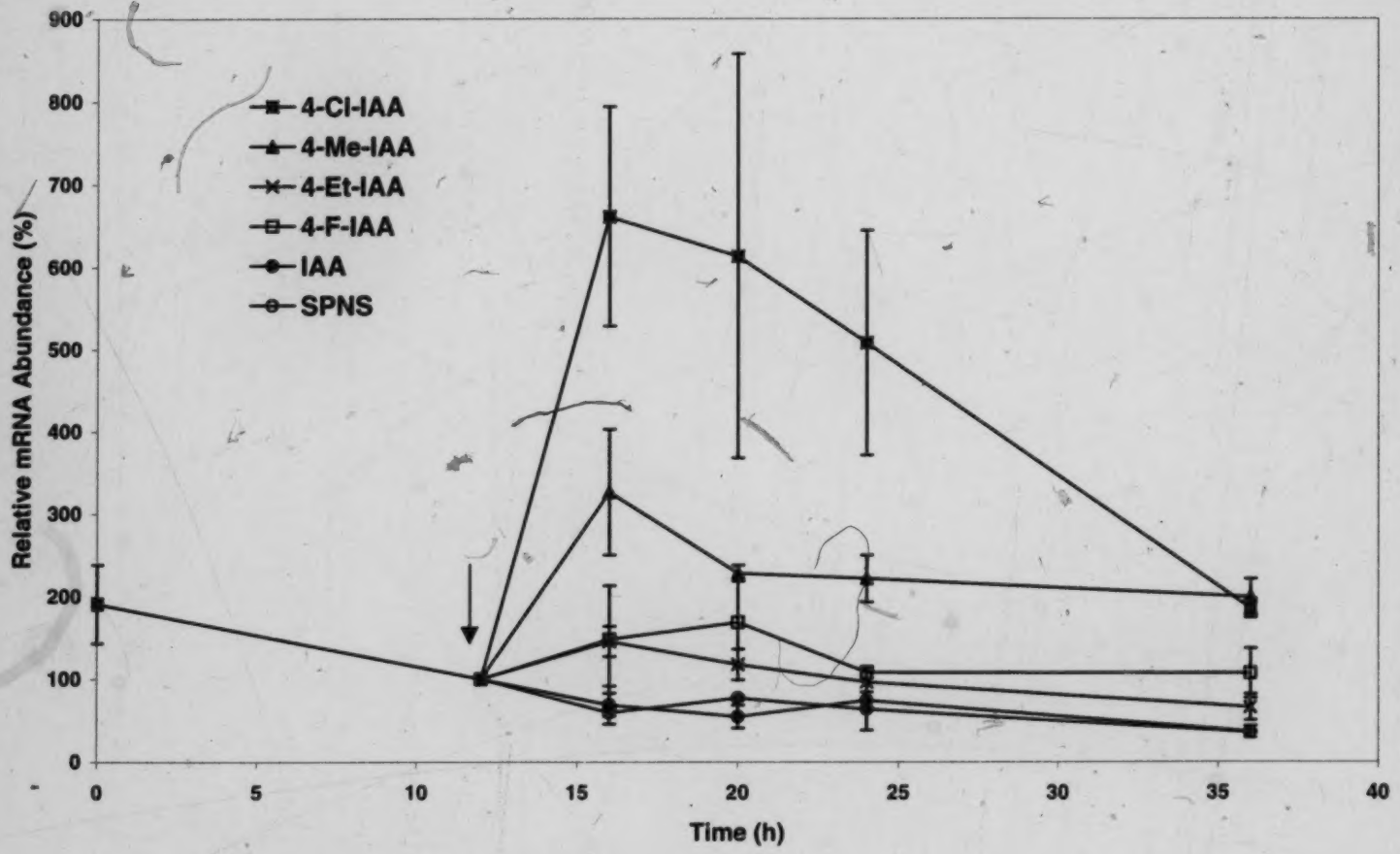

300
250
200
150
100
50
0
Relative mRNA Abundance (%)
SP
SPNS
0
5
10
15
20
25
30
35
40
Time (h)

Figure 2-9. Relative mRNA abundance of GA 20-oxidase transcripts in deseeded pea pericarps treated with 4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA, IAA or 0.1% Tween 80 (SPNS) as described in Figure 2-7. Autoradiograms were scanned with an imaging densitometer and these values were normalized to the value for pericarps at the time of hormone application (12 h after deseeding). Data are means ± SE, n = 3, with one exception; for the IAA treatment, n = 2.


4-Cl-IAA
4-Me-IAA
4-Et-IAA
4-F-IAA
IAA
SPNS
Relative mRNA Abundance (%)
Time (h)
0
100
200
300
400
500
600
700
800
900
0
5
10
15
20
25
30
35
40

GA 20-oxidase mRNA levels from deseeded pericarps increased linearly with increasing log concentration of applied 4-Cl-IAA 4 h after application ($P < 0.01$ Student's T value; $r = 0.873$; Figures 2-6, 2-10 and 2-11). Transcript levels were highest in deseeded pericarps treated with 100μM 4-Cl-IAA 4 h after hormonal application (7 times higher than levels observed in SPNS control), and the transcript level remained significantly elevated compared to all other treatments for up to 12 h after hormonal application (Figures 2-10 and 2-11). Application of 50 μM 4-Cl-IAA to deseeded pericarps increased GA 20-oxidase levels 4.1 times above the SPNS control, and the transcript levels remained above pericarps treated with 10 and 1 μM 4-Cl-IAA and the SPNS control for up to 12 h after treatment (Figures 2-10 and 2-11). Four hours after application of 10 and 1 μM 4-Cl-IAA to deseeded pericarps, GA 20-oxidase mRNA levels increased 3 and 1.7 times above the SPNS control, respectively. However, 10 and 1 μM 4-Cl-IAA application resulted in little to increase in pericarp GA 20-oxidase transcript levels above the SPNS control after the initial increase 4 h after application with one exception (10 μM 4-Cl-IAA 24 h after treatment; Figures 2-10 and 2-11).

Representative blots were reprobed with an *Arabidopsis thaliana* actin riboprobe as a developmental control (used as a marker for the general mRNA population) to determine if treatment effects were specific to pericarp GA 20-oxidase mRNA or if they were due to a general trend in the total mRNA population. GA 20-oxidase gene expression patterns (over the 24-h treatment period) for deseeded pericarps treated with the 4-substituted auxins and 4-Cl-IAA from 1 to 100 μM (Figures 2-7 and 2-10) were not similar to the actin gene expression patterns.

2.4 Discussion

GA 20-oxidase mRNA levels and growth of pea pericarp were maintained when seeds were present and substantially decreased after seed removal (Figures 2-3, 2-7 and 2-8). These

Figure 2-10. Time course of GA 20-oxidase mRNA accumulation in deseeded pea pericarp treated with 4-Cl-IAA at 100, 50, 10, 1, or 0 µM (0.1% Tween 80; SPNS). Two DAA pericarps were split and deseeded and 30 µL of 4-Cl-IAA was applied 12 h after deseeding. rRNA banding in a representative gel (100 µM 4-Cl-IAA treatment) is shown prior to membrane transfer (visualized by staining with ethidium bromide). The 18S rRNA band was used as a loading control for all samples and an actin probe was used as a developmental control for one replication of all treatments.

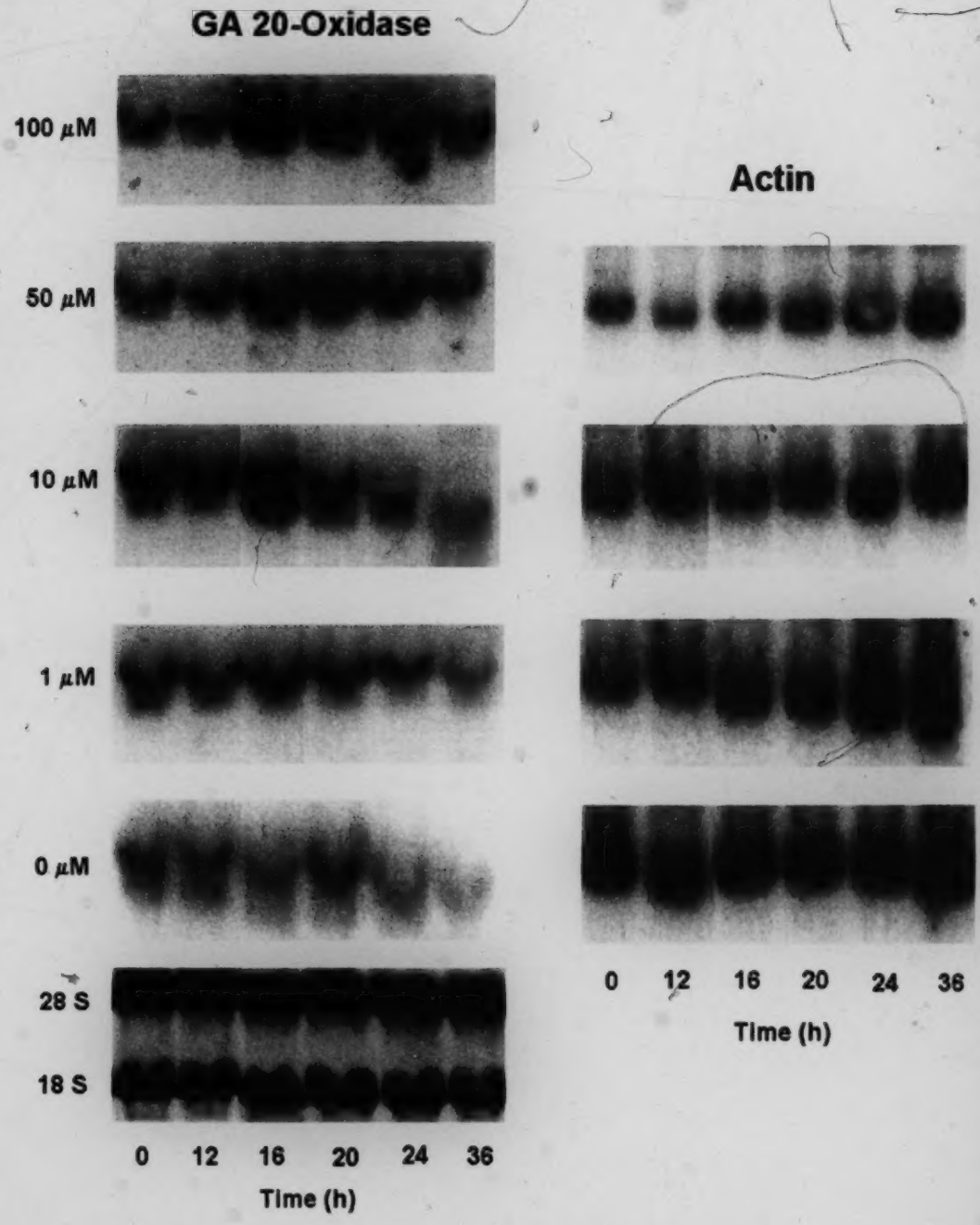
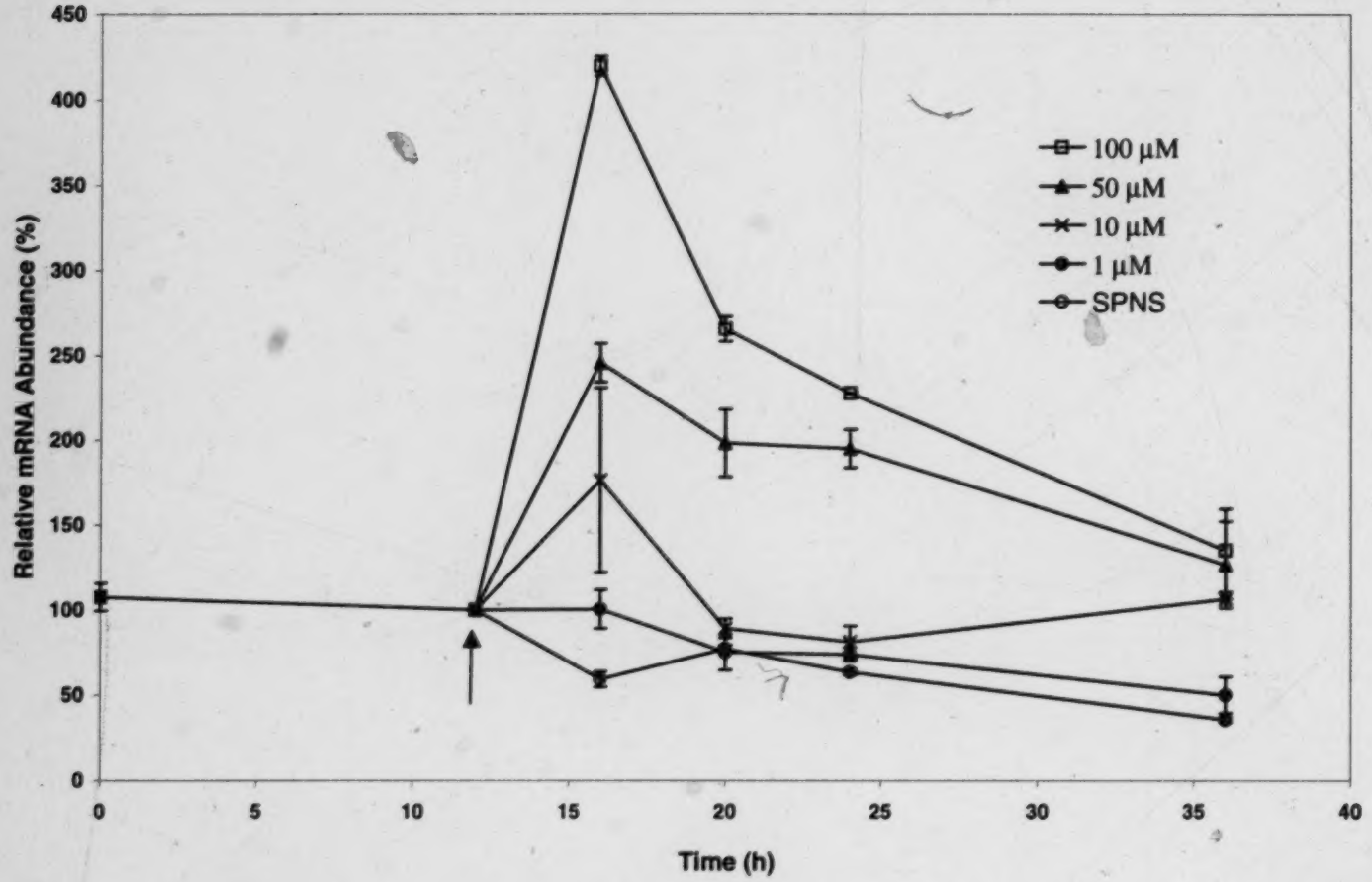

GA 20-Oxidase
100 μM
50 μM
10 μM
1 μM
0 μM
28 S
18 S
0 12 16 20 24 36
Time (h)
Actin
0 12 16 20 24 36
Time (h)

Figure 2-11. Relative mRNA abundance of GA 20-oxidase transcripts of deseeded pea pericarp treated with 4-Cl-IAA at 100, 50, 10, 1 or 0 μM (0.1% (v/v) Tween 80; SPNS) as described in Figure 2-10. Autoradiograms were scanned with an imaging densitometer and values were normalized to the value for pericarps at the time of hormone application (12 h after deseeding). The arrow indicates the time of hormone application. Data are means ± SE, n = 2, with one exception; for the SPNS treatment, n = 3.


Relative mRNA Abundance (%)
Time (h)
100 µM
50 µM
10 µM
1 µM
SPNS
0
50
100
150
200
250
300
350
400
450
0
5
10
15
20
25
30
35
40

data agree with those of van Huizen et al. (1997) and demonstrate that seeds are required to maintain GA 20-oxidase mRNA levels for normal GA biosynthesis in the pericarp tissue. These findings are also consistent with the previous results from our group (Ozga et al., 1992; van Huizen et al., 1995) that the activity of the enzyme oxidizing GA_{19} to GA_{20} was maintained in pericarps with seeds and decreased to minimal levels after deseeding.

4-Cl-IAA significantly increased growth and mRNA levels of GA 20-oxidase in deseeded pericarp (Figures 2-2, 2-3, 2-7 and 2-9). These data are in agreement with the work of Reinecke et al. (1995) and van Huizen et al. (1997). However, in the previous work by van Huizen et al. (1997) it was not known if the effect of 4-Cl-IAA on GA 20-oxidase mRNA levels in the pericarp was linked to auxin-induced growth or a non-specific effect attributable to auxin-type molecules. To further understand how auxin regulates GA biosynthesis in pea pericarp, we used 4-substituted auxins that possess a range of biological activities in the split-pericarp assay system (Reinecke et al., 1999) as molecular tools to determine the specificity of auxin regulation of GA 20-oxidase expression.

In this study, 24 h after hormone treatment the lengths of deseeded pericarps treated with 4-Cl-IAA were significantly greater than pericarps treated with any of the other auxins tested or the SPNS control (Figure 2-2). The growth promoting activities of the 4-substituted auxins 7 days after the initial hormone treatment (50 μM) when first applied 12 h after deseeding (4-Cl-IAA > 4-Me-IAA > 4-F-IAA > 4-Et-IAA ≥ 4-H-IAA; Figure 2-2) were similar to those reported by Reinecke et al. (1999; hormone solutions were added immediately after deseeding) in the pea split-pericarp system with one exception (4-Et-IAA was less active when applied 12 h after deseeding). This observed difference in 4-Et-IAA growth is most likely the result of differences in applied dose to the pea pericarp. Reinecke et al. (1999) observed moderate 4-Et-IAA growth in pea pericarp only at higher concentrations of exogenous auxin (100 μM). Previous work by

Reinecke et al. (1995; 1999) on the structure-activity relationships among these 4-substituted auxins suggested that the size and lipophilicity of the 4-substituent are important factors determining biological activity in the pea split-pericarp growth assay. Any relationship between molecular structure and auxin activity at the whole-organ level reflects the rate-determining steps of a complex response mechanism. One possible step leading to pericarp growth is the up-regulation of GA 20-oxidase expression in the pericarp. The ability of the 4-substituted auxins (at 50 μM) to increase the levels of GA 20-oxidase mRNA was associated with their ability to stimulate pericarp growth. The greatest increase in pericarp GA 20-oxidase mRNA levels and growth were observed when deseeded pericarps were treated with the naturally occurring auxin, 4-Cl-IAA (Figures 2-3, 2-7 and 2-9). 4-Me-IAA was the second most biologically active auxin for both stimulation of GA 20-oxidase mRNA levels and pericarp growth (Figures 2-3, 2-7 and 2-9). Application of the 4-substituted IAA analogues, 4-Et-IAA and 4-F-IAA as well as the naturally occurring auxin IAA, to deseeded pericarps resulted in minimal or no increase in GA 20-oxidase transcript levels as well as pericarp growth (Figures 2-3, 2-7 and 2-9). These results provide evidence that regulation of GA 20-oxidase expression by auxin is specific to the biologically active auxins in the split-pericarp assay system. In addition, of the two naturally occurring auxins in pea fruit (4-Cl-IAA and IAA), only 4-Cl-IAA increased pericarp growth and GA 20-oxidase mRNA levels. These results are consistent with the hypothesis that 4-Cl-IAA has a specific role as a seed signal involved in the coordination of growth and development of seeds and the surrounding pericarp tissue in pea (Reinecke, 1999). The association of biological activity (pericarp growth and stimulation of GA 20-oxidase mRNA levels) with the indole-ring substituents's size, its lipophilicity, and its location on the indole ring (pericarp growth; Reinecke et al., 1995) suggests a novel receptor and/or signal transduction pathway for 4-Cl-IAA in pea pericarp growth.

We have also shown that 4-Cl-IAA has concentration dependent stimulatory effects on pericarp growth and GA 20-oxidase transcript levels, with the effect decreasing with decreasing 4-Cl-IAA concentration (Figures 2-4, 2-5, 2-6, 2-9, and 2-10). Slight differences however are observed between 50 and 100 µM 4-Cl-IAA treatments (Figure 2-5) where pericarp growth patterns are equivalent but GA 20-oxidase mRNA accumulation is significantly higher at 100 µm 4-Cl-IAA. A possible explanation for this observed difference is the time of hormone application. Reinecke et al. (1999) observed dose-dependent pericarp growth differences between 50 and 100 µM 4-Cl-IAA, however, the time of hormone application was immediately after removal of seeds from the pericarp. The delay in hormone application with our results may have resulted in effects of ethylene on the general physiological response of the pericarp that was not affected at the mRNA level. In general, these data agree with growth studies by Reinecke et al. (1995, 1999) and demonstrate 4-Cl-IAA is biologically active (increase in pericarp growth and GA 20-oxidase mRNA levels) over a wide range of concentrations in pea pericarp and supports the hypothesis that 4-Cl-IAA modulates pea fruit growth in part by stimulating GA biosynthesis (production of GA_{20}).

We now have evidence that seeds, GAs, and auxin (4-Cl-IAA) can regulate growth, *in vivo* activity of the enzyme oxidizing GA_{19} to GA_{20} (Ozga et al., 1992; van Huizen et al., 1995), and GA 20-oxidase gene expression in pea pericarp (van Huizen et al., 1996; van Huizen et al., 1997). Furthermore, in this study we demonstrated that regulation of GA 20-oxidase expression by auxin is specific to the biologically active auxins in the split-pericarp assay system and that stimulation of GA 20-oxidase transcript levels by 4-Cl-IAA is dose dependent. Future research is required to determine whether the auxin growth responses and GA 20-oxidase expression are in part due to the differential effects of these 4-substituted auxins on ethylene biosynthesis and/or ethylene action. These future studies would provide further invaluable information in understanding the interaction of auxin and GA in fruit growth.

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Chapter 3

Gibberellin Metabolism in Pea Pericarp

3.1 Introduction

Pea fruit are being studied as a model system to understand coordination of seed and ovary growth during early fruit development. Normal pea pericarp growth requires the presence of seeds, removal or destruction of seeds results in reduced pericarp growth and subsequent abscission (Eeuwens and Schwabe, 1975). Developing pea seeds and pericarps contain GAs (GA_1 and GA_3; García-Martinez et al., 1991; Rodrigo et al., 1997) as well as auxins (4-Cl-IAA and IAA; Marumo et al., 1968; Magnus et al., 1997). The requirement of seeds for pericarp growth can be replaced by the application of GAs (Eeuwens and Schwabe, 1975) and the naturally occurring auxin 4-Cl-IAA (Reinecke et al., 1995, 1999). However, the other naturally occurring auxin in pea fruit, IAA, inhibits growth in deseeded pericarps (Reinecke et al., 1995).

Studies by Reinecke et al. (1995) have investigated the importance of the substituent at the 4-position of the indole ring in maintaining pea pericarp growth. Initial work determined the growth promoting properties of 4-, 5-, 6- and 7-chloro-IAA's as well as the corresponding fluoro-IAA analogues in deseeded pea pericarp. This work demonstrated that the 4-substituent of the indole ring was important for biological activity in pea pericarp growth. To further elucidate the role of auxin in pea pericarp growth Reinecke et al. (1995) compared the growth-promoting response and the physiochemical properties of 4-Cl-IAA and its 4-substituted analogues: 4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA and IAA. The comparative ability of these auxins to stimulate pea pericarp growth was: 4-Cl-IAA ≥ 4-Me-IAA > 4-Et-IAA > 4-F-IAA ≥ IAA (similar growth promoting activities were observed by Ngo (Chapter 2). The 4-substituent's size and its lipophilicity were associated with the growth promoting activity of pea pericarps (Reinecke et al., 1999).

It has been hypothesized that seeds may promote pericarp growth by maintaining GA biosynthesis in the pericarp (Sponsel, 1982). The early 13-hydroxylation pathway of GA biosynthesis is known to occur in pea seeds (Graebe, 1987): $GA_{12} \rightarrow GA_{53} \rightarrow GA_{44} \rightarrow GA_{19} \rightarrow GA_{20} \rightarrow GA_1$. Pea pericarps have been shown to metabolize [^{2}H]GA_{53} to [^{2}H]GA_1, [^{14}C]GA_{12} to [^{14}C]GA_{20}, and [^{14}C]GA_{19} to [^{14}C]GA_{20}, when seeds are present (Maki and Brenner, 1991; Ozga et al., 1992; van Huizen et al., 1995). Removal of seeds inhibited pericarp growth as well as the conversion of [^{14}C]GA_{12} to [^{14}C]GA_{20} (Ozga et al., 1992) and [^{14}C]GA_{19} to [^{14}C]GA_{20} (van Huizen et al., 1995). These data suggest that the seeds regulate a key step in the GA biosynthesis pathway, i.e. conversion of GA_{19} to GA_{20}.

Using the split-pericarp assay system, van Huizen et al. (1997) observed that GA 20-oxidase transcript levels in 2 DAA pericarp with seeds remained relatively stable throughout the 36-h treatment period; however, when the seeds were removed the pericarp transcript levels declined. When 2 DAA deseeded pericarps were treated with 4-Cl-IAA, a significant increase in GA 20-oxidase mRNA levels was detected within 2 h and transcript levels remained elevated for up to 12 h after 4-Cl-IAA application. In addition, van Huizen et al. (1997) found that application of GA_3 to deseeded pericarps decreased GA 20-oxidase mRNA levels. These data suggest that 4-Cl-IAA regulates GA 20-oxidase gene expression in young pea pericarps and support the view that bioactive GAs control their own synthesis through end-product repression of GA 20-oxidase gene expression. Simultaneous application of GA_3 and 4-Cl-IAA to deseeded pericarp delayed the decrease of GA 20-oxidase expression compared to GA_3 alone, demonstrating an interaction of these hormones in the regulation of GA 20-oxidase steady state mRNA levels (van Huizen et al., 1997).

Ngo et al. (1998, 1999, Chapter 2) reported on the effects of the 4-substituent of the indole ring on maintaining GA 20-oxidase mRNA levels in the absence of seeds in pea pericarp. The ability of the 4-substituted auxins (at 50 μM) to increase GA 20-oxidase mRNA was

associated with their ability to stimulate pericarp growth. The greatest increase in pericarp GA 20-oxidase mRNA levels and growth were observed when deseeded pericarps were treated with 4-Cl-IAA. 4-Me-IAA was the second most biologically active auxin for both stimulation of GA 20-oxidase mRNA levels and pericarp growth. Application of the 4-substituted IAA analogues, 4-Et-IAA and 4-F-IAA as well as IAA, to deseeded pericarps resulted in minimal or no increase in GA 20-oxidase transcript levels as well as pericarp growth. These results are consistent with the hypothesis that 4-Cl-IAA has a specific role as a seed signal involved in the coordination of growth and development of seeds and the surrounding pericarp tissue in pea (Reinecke, 1999).

To determine if the pattern of GA 20-oxidase mRNA expression exhibited by pericarp treated with the 4-substituted auxins is correlated with *in vivo* GA 20-oxidase conversion of GA_{19} to GA_{20} and to further understand how seeds and auxin regulate GA biosynthesis in pea pericarp, we used the 4-substituted auxins that possess a range of biological activities in the split-pericarp assay system as tools to determine the specificity of auxin regulation of [^{14}C]GA_{19} metabolism. In addition, we also investigated the effects of the *sln* mutation (reported to impair the conversion of GA_{20} to GA_{29} in young pea pericarps, resulting in reduced levels of GA_{29} and higher levels of GA_{20} and GA_1 in this tissue; MacKenzie-Hose et al., 1998) on seed and 4-Cl-IAA induced growth and [^{14}C]GA_{19} metabolism in pea pericarps. These studies show that the conversion of GA_{19} to GA_{20} is specifically regulated by the biologically active auxins in the split-pericarp assay system and that GA_3 inhibits conversion of GA_{20} to GA_{29}.

3.2 Methods and Materials

3.2.1 Plant Material and Treatments

Plants of *Pisum sativum* L., line I_3 were grown as previously described (van Huizen et al., 1995). One fruit from the third and fifth flowering node was treated per plant, and subsequent flowers were removed as they developed. Terminal apical meristems of plants were left intact and pericarp remained attached to the plant for the entire experiment. Removal of seeds was carried out using a split-pericarp technique as described by Ozga et al. (1992). Briefly, 2 DAA pericarps measuring in length from 15-20mm were split down the dorsal suture and seeds were either left intact (SP treatment) or removed (SPNS treatment). Splitting of the pericarp and removal of seeds were completed 12 h prior to all hormone applications and 24 h prior to radiolabel application. Pericarps were treated with 4-Cl-IAA, 4-Me-IAA, IAA, GA_3, and GA_3 plus 4-Cl-IAA (30 µL, 50 µM in 0.1% (v/v) Tween 80). All solutions were applied to the inside surface of the pericarp wall (endocarp); SP and SPNS controls were treated with 0.1% (v/v) Tween 80. Plastic bags were used to cover treated pericarps to maintain high humidity (Figure 3-1).

Plants of genotype SLN (Torsdag WT; J I992) and *sln* (JI3011) were also grown as described above from flowering nodes 2 to 8. SLN and *sln* pericarps were deseeded and treated with 4-Cl-IAA as described above (30 µL, 50 µM in 0.1% (v/v) Tween 80) and SP and SPNS controls were treated with 0.1% (v/v) Tween 80.

17-[^{14}C]GA_{19} (specific activity of 54 mCi/mmol) was applied (60,000 dpm per pod in 5 µL of 50% (v/v) aqueous methanol) 12 h after hormonal application (24 h after deseeding) to the inside surface of the pericarp wall (endocarp). Treated pericarps (seeds were removed if present) were harvested onto dry ice 4, 12 and 24 h after [^{14}C]GA_{19} application and stored at –80°C until extraction.

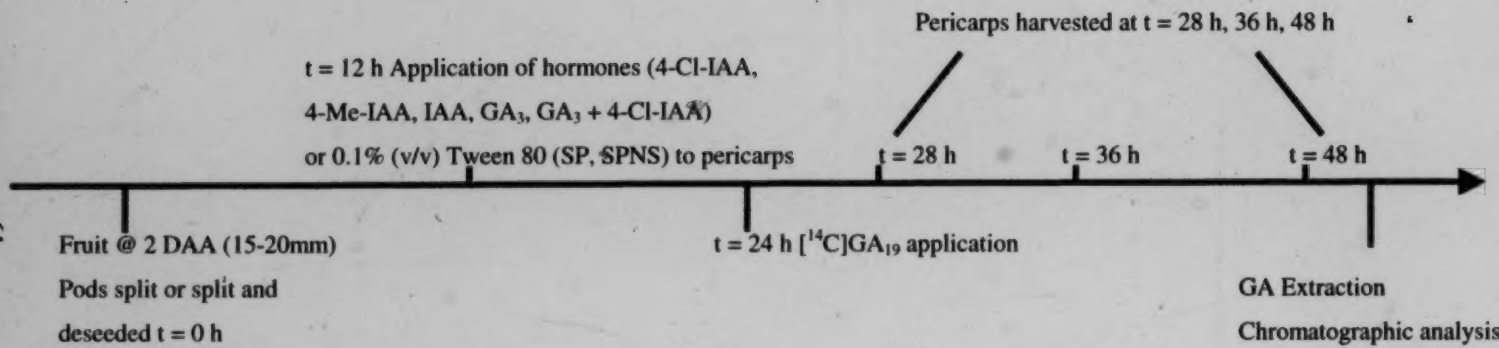

Pericarps harvested at t = 28 h, 36 h, 48 h
t = 12 h Application of hormones (4-Cl-IAA,
4-Me-IAA, IAA, GA3, GA3 + 4-Cl-IAA)
or 0.1% (v/v) Tween 80 (SP, SPNS) to pericarps
t = 28 h
t = 36 h
t = 48 h
Fruit @ 2 DAA (15-20mm)
Pods split or split and
deseeded t = 0 h
t = 24 h [14C]GA19 application
GA Extraction
Chromatographic analysis

Figure 3-1. Time course of hormone and [^{14}C]GA_{19} application to pea pericarps.

3.2.2 Extraction Procedure and Partitioning

Using a Polytron homogenizer, radiolabeled pericarps (three per sample) were homogenized in silylated 30 mL corex tubes with 10 mL cold 80% (v/v) methanol containing 10 mg/L butylated hydroxytoluene. As an external standard, 10,000 dpm of 17-[^{14}C]-GA_7 was added at the time of homogenization for determination of radioactive metabolite recovery. After homogenization, samples were gently shaken in darkness at 4°C for 12-16 h then centrifuged at 10,000g for 30 minutes. The supernatant was removed and the residue was resuspended in 10 mL of the homogenization solvent and gently shaken at 4°C in darkness for at least 4 h. The residue extracts were centrifuged at 10,000g for 30 minutes, and the combined supernatants were reduced to the aqueous phase using a vacuum concentrator (Savant, Farmingdale, NY). The pH of the aqueous extracts was adjusted to 8.0 with NH_4OH (0.1N) and partitioned against n-hexane (5mL) four times in silylated 20 mL glass scintillation vials. The aqueous fraction was then adjusted to pH 3.0 with 0.1N HCl and partitioned against ethyl acetate (5 mL) five times. The combined ethyl acetate extracts were reduced to approximately 3mL using a vacuum concentrator and partitioned against 5% (w/v) aqueous $NaHCO_3$ (5mL) four times. The combined $NaHCO_3$ extracts were transferred to 30 mL silylated pyrex tubes, the pH was adjusted to 3.0 with concentrated HCl on ice, and partitioned against ethyl acetate (5mL) five times. The ethyl acetate extracts were combined and evaporated to near dryness and transferred to 7 mL silylated scintillation vials and dried down under vacuum.

3.2.3 Chromatography

The ethyl acetate extracts were resuspended in 400 µL of 20% methanol, passed through 0.45-µm nylon filters prior to injection onto a 4.5 x 250 mm Spherisorb C_{18} column (5µm; Beckman α). The samples were eluted at a flow rate of 1.0 mL/min using a linear gradient of

0.01% TFA (solvent A) and 100% methanol (solvent B). Conditions of the linear gradient were 20% solvent B for 1 min, gradient to 100% solvent B in 45 min, and isocratic 100% solvent B for 5 min. Radioactivity in the effluent was monitored using a flow-through radiochemical detector (Beckman 171). Radioactive fractions eluting near standard retention times of GA_8 (9.2 min), GA_{29} (12.6 min), GA_3 (16.3 min), GA_1 (17.8 min), GA_5 (25.0 min), GA_{20} (26.4 min), GA_{19} (29.2 min) and GA_7 (31.0 min) were collected and dried down. Collected ^{14}C-GAs were methylated using diazomethane and rechromatographed as their methyl esters by C_{18}–HPLC using the same solvent system.

3.3 Results

3.3.1 Hormone Stimulated Pericarp Growth

The length of 2 DAA I_3 (Alaska-type) pericarps with seeds (SP) and deseeded pericarps treated with 50 μM auxin (4-Cl-IAA, 4-Me-IAA, IAA), GA_3, GA_3 plus 4-Cl-IAA, or 0.1% (v/v) Tween 80 (SPNS) increased with time over the 24 h [^{14}C]GA_{19} incubation period (Figure 3-2). Twenty-four h after [^{14}C]GA_{19} application, growth of pericarps with seeds (SP) and deseeded pericarps treated with GA_3 plus 4-Cl-IAA, and GA_3 was the greatest followed by 4-Cl-IAA. Moderate growth was observed in 4-Me-IAA-treated pericarps 24 h after [^{14}C]GA_{19} application, whereas IAA-treated pericarps did not grow significantly compared to the SPNS control (Figure 3-2).

Pericarps from the SLN (WT) genotype responded similarly to deseeding and 4-Cl-IAA treatment as the I_3 (Alaska-type) cultivar (Figure 3-3A). Twenty four hours after [^{14}C]GA_{19} application, growth of SLN pericarps with seeds (SP) was the greatest (1.8 times greater than SPNS control) followed by deseeded pericarps treated with 4-Cl-IAA (1.3 times greater than SPNS). Deseeded SLN pericarps treated with 0.1% (v/v) Tween 80 (SPNS) did not grow during

Figure 3-2. The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, IAA, GA_3 and GA_3 plus 4-Cl-IAA on pea pericarp growth over 36 h. Two DAA pericarps were split or split and deseeded and 30 µL of 50 µM hormone or 0.1% (v/v) Tween 80 (SP and SPNS) was applied 12 h after deseeding. The line arrow indicates the time of [^{14}C]GA_{19} application. Data are means ± SE, n = 9.

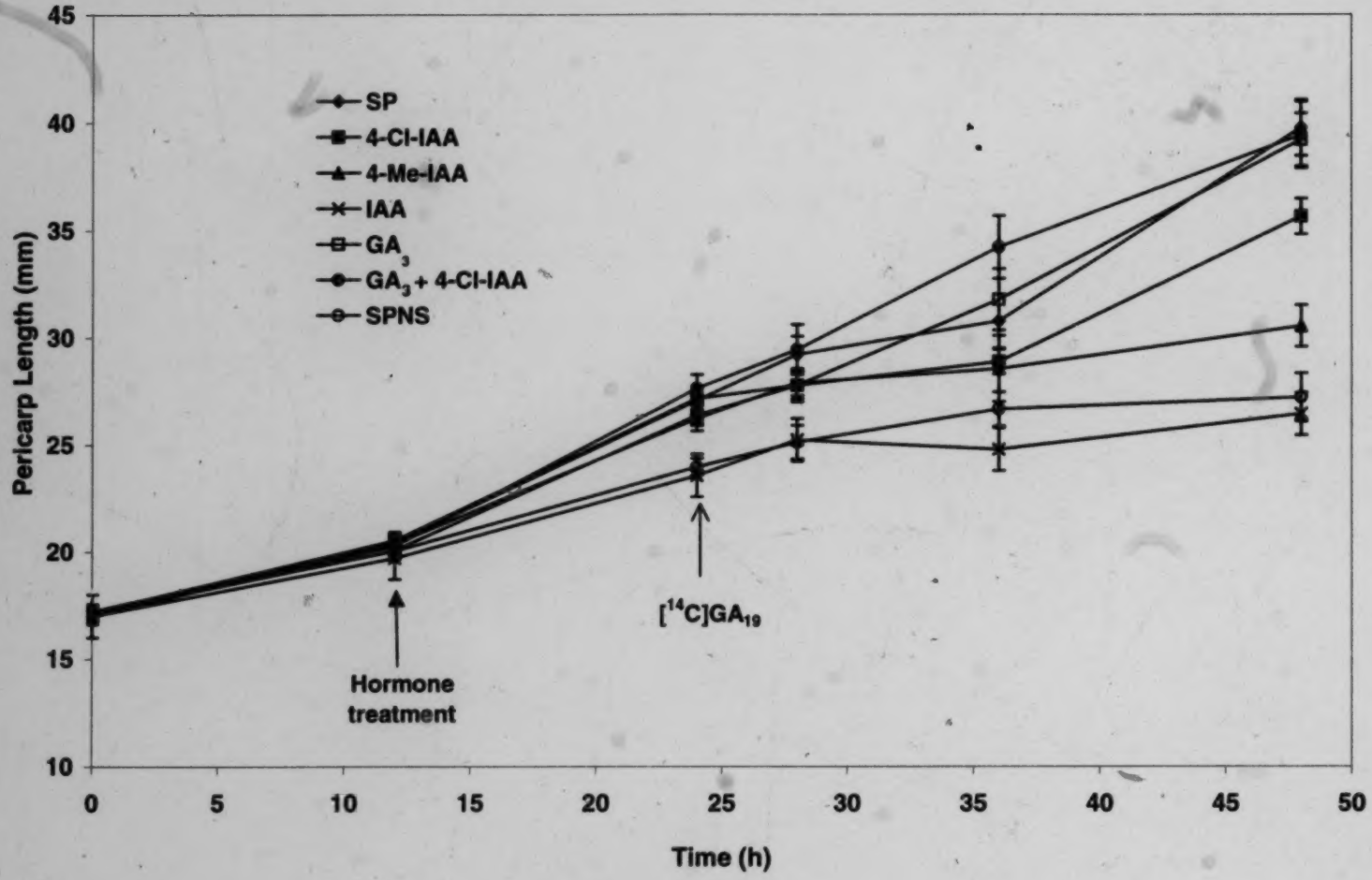
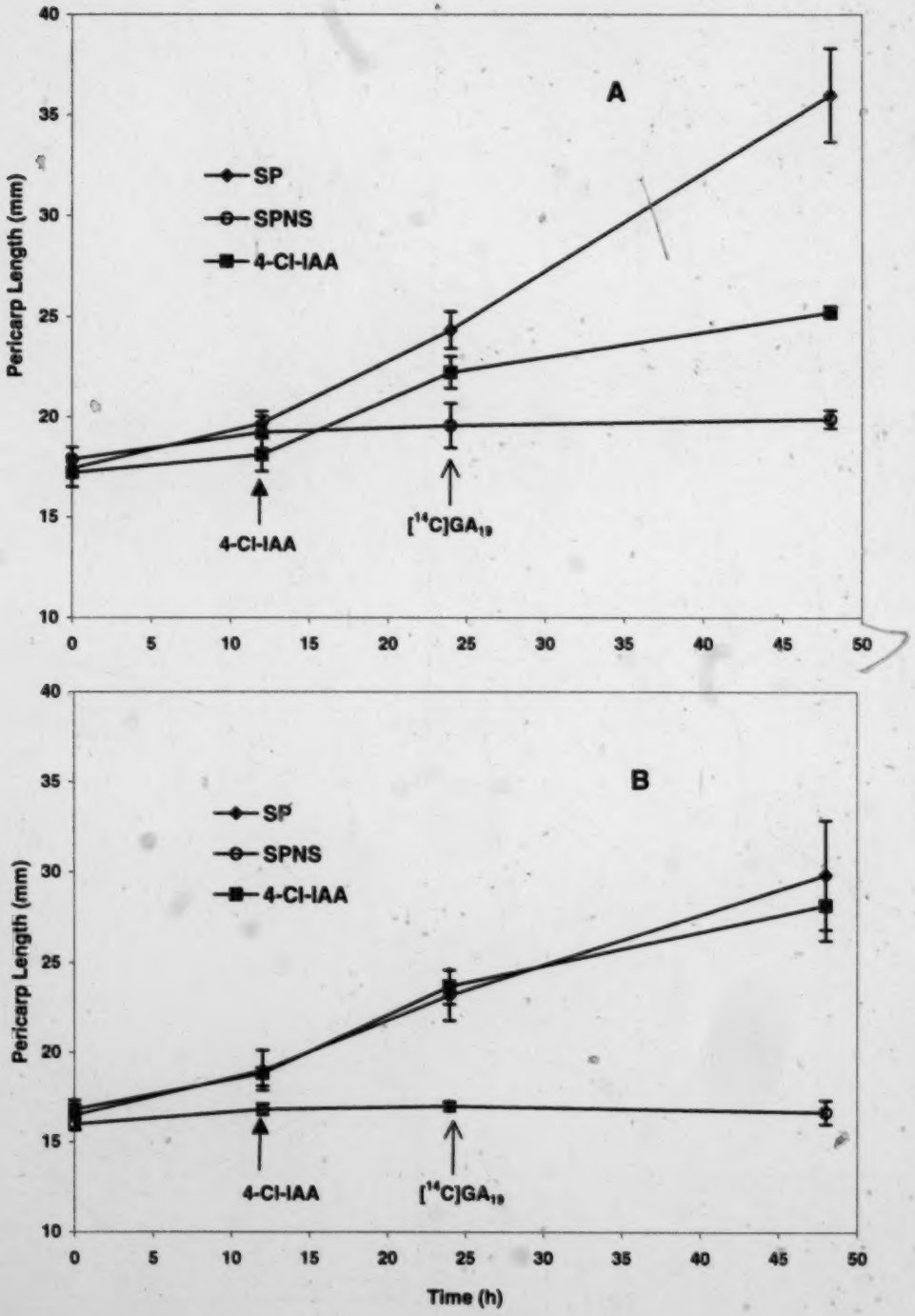

SP
4-Cl-IAA
4-Me-IAA
IAA
GA3
GA3 + 4-Cl-IAA
SPNS
Pericarp Length (mm)
Time (h)
Hormone treatment
[14C]GA19
10
15
20
25
30
35
40
45
0
5
10
15
20
25
30
35
40
45
50

Figure 3-3. The effect of seeds (SP), seed removal (SPNS), and seed removal plus auxin treatment with 4-Cl-IAA on pea pericarp growth of WT; SLN (A) and *sln* (B). Two DAA pericarp were split or split and deseeded and 30 µL of 50 µM 4-Cl-IAA or 0.1% (v/v) Tween 80 (SP and SPNS) was applied 12 h after deseeding. The line arrow indicates the time of [^{14}C]GA_{19} application. Data are means ± SE, n = 9.


A
SP
SPNS
4-Cl-IAA
Pericarp Length (mm)
4-Cl-IAA
[14C]GA19
B
SP
SPNS
4-Cl-IAA
Pericarp Length (mm)
4-Cl-IAA
[14C]GA19
Time (h)

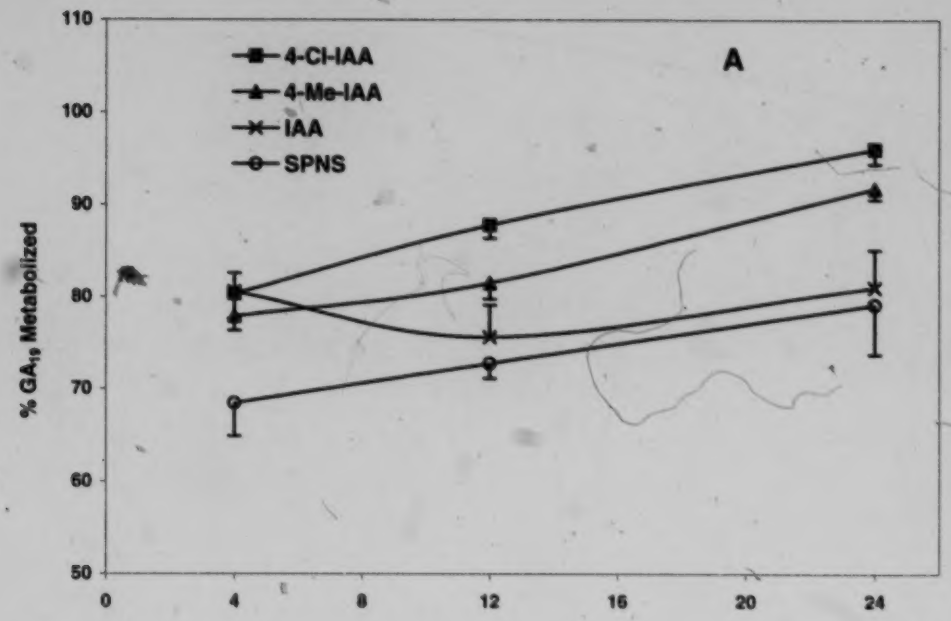
the experiment (Figure 3-3A). The *sln* pericarps with seeds (SP) and deseeded *sln* pericarps treated with 4-Cl-IAA grew similarly, obtaining a length 1.75 times greater than the SPNS control 24 h after [^{14}C]GA_{19} application (Figure 3-3B).

3.3.2 [^{14}C]GA_{19} metabolism in pea pericarp

Four h after [^{14}C]GA_{19} application, deseeded pericarps treated with auxins (4-Cl-IAA, 4-Me-IAA, and IAA) had metabolized [^{14}C]GA_{19} to a greater extent than pericarps with seeds (SP) and deseeded pericarps treated with GA_3, GA_3 plus 4-Cl-IAA, and 0.1% Tween 80 (SPNS) (Figures 3-4A and B). The amount of [^{14}C]GA_{19} metabolized by the pericarp significantly increased at both 12 and 24 h incubation periods in deseeded pericarps treated with 4-Cl-IAA and 4-Me-IAA, resulting in the highest amount of [^{14}C]GA_{19} metabolized (after a 24 h incubation period) among all treatments. An increase in the percentage of [^{14}C]GA_{19} metabolized occurred after 24 h of radiolabel incubation with pericarps with seeds (SP) and deseeded pericarps treated with GA_3, GA_3 plus 4-Cl-IAA, and 0.1% (v/v) Tween 80 (SPNS) (Figure 3-4B). No further metabolism of [^{14}C]GA_{19} was detected in deseeded pericarps treated with IAA after 4 h of radiolabel incubation.

The amount of [^{14}C]GA_{20} present in pericarps with seeds (SP) was maintained at a moderate level throughout the 24 h radiolabel incubation period (Figure 3-5B). Seed removal (SPNS) resulted in significantly lower accumulation of [^{14}C]GA_{20} in pericarps and the amount of [^{14}C]GA_{20} present in deseeded pericarp decreased with increasing radiolabel incubation time (Figure 3-5B). Accumulation of [^{14}C]GA_{20} 4 h after [^{14}C]GA_{19} application was greatest in deseeded pericarp treated with 4-Cl-IAA compared to all other treatments (Figures 3-5A). Among the auxin treatments, [^{14}C]GA_{20} accumulation was the highest 4 h after [^{14}C]GA_{19} application and decreased with increasing radiolabel incubation time with 4-Cl-IAA stimulating

Figure 3-4. A) The effect of seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, and IAA, and B) the effect of seeds (SP), seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, GA_3, and GA_3 plus 4-Cl-IAA on the percentage of [^{14}C]GA_{19} metabolized over a 24 h period. Pericarp at 2 DAA were split or split and deseeded and 30 µL of 50 µM hormone or 0.1% (v/v) Tween 80 (SP and SPNS) was applied 12 h after deseeding. [^{14}C]GA_{19} was applied 24 hours after deseeding. Data are means ± SE, n = 3.


A
4-Cl-IAA
4-Me-IAA
IAA
SPNS
% GA19 Metabolized
110
100
90
80
70
60
50
0
4
8
12
16
20
24

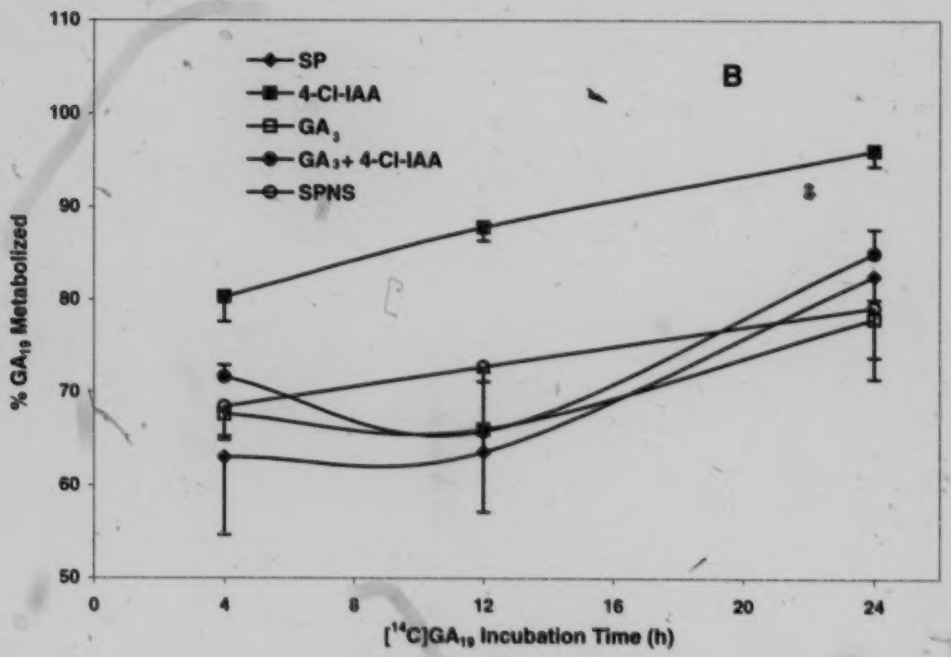
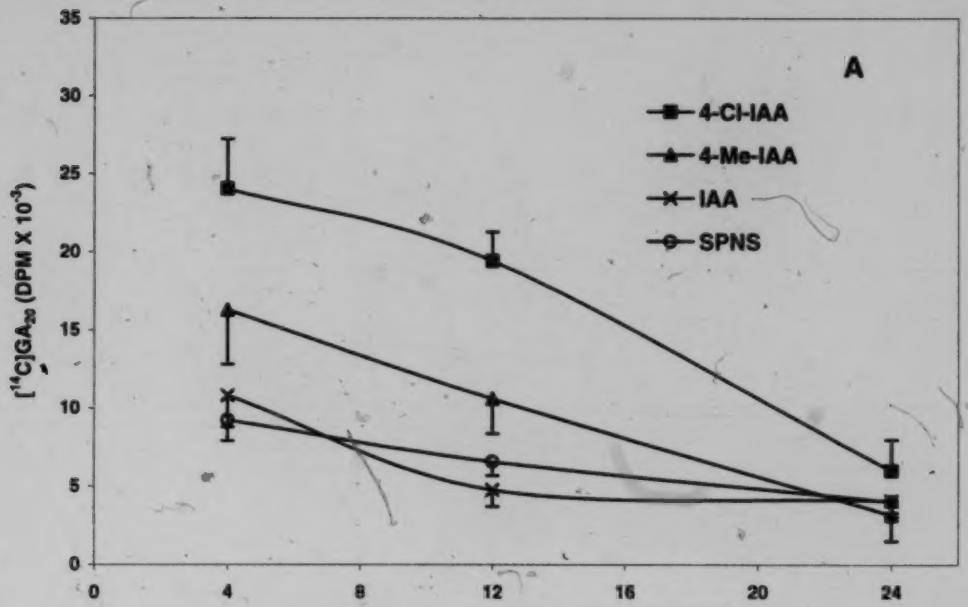
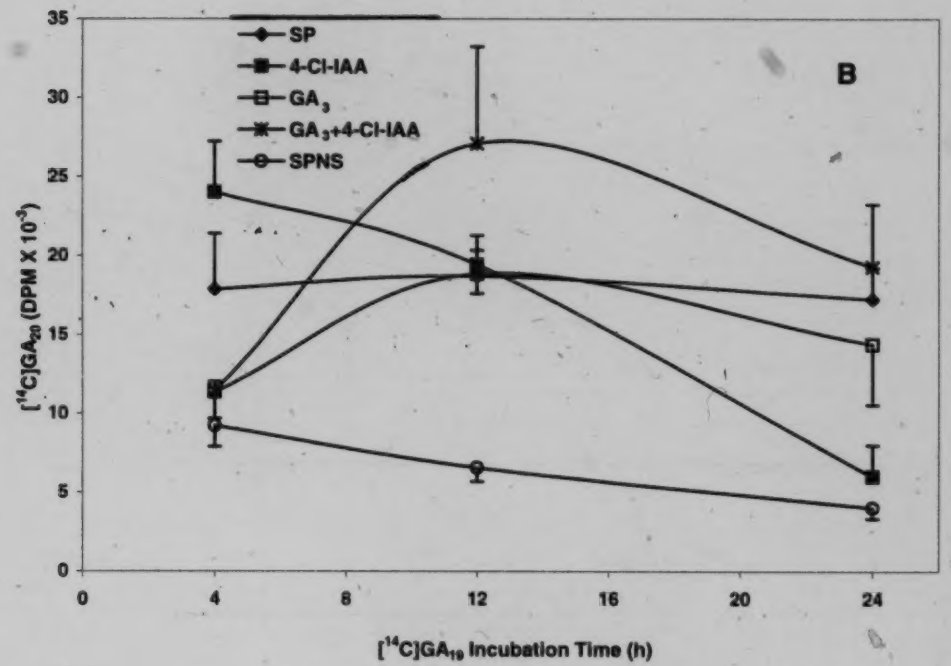

B
SP
4-Cl-IAA
GA3
GA3+ 4-Cl-IAA
SPNS
% GA19 Metabolized
110
100
90
80
70
60
50
0
4
8
12
16
20
24
[14C]GA19 Incubation Time (h)

Figure 3-5. A) The effect of seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, and IAA and B) the effect of seeds (SP), seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, GA_3, and GA_3 plus 4-Cl-IAA on $[^{14}C]GA_{20}$ accumulation over a 24 h period. Pericarps treated as described in Figure 3-4. Data are means ± SE, n = 3.


A
4-Cl-IAA
4-Me-IAA
IAA
SPNS
[14C]GA20 (DPM X 10-3)
0
5
10
15
20
25
30
35
0
4
8
12
16
20
24

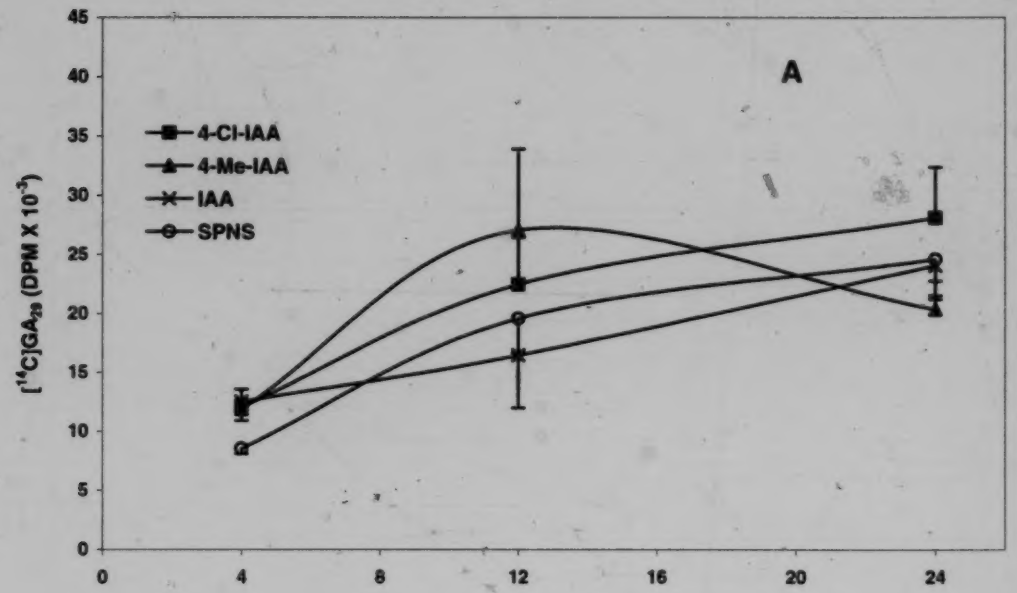

B
SP
4-Cl-IAA
GA3
GA3+4-Cl-IAA
SPNS
[14C]GA20 (DPM X 10-3)
0
5
10
15
20
25
30
35
0
4
8
12
16
20
24
[14C]GA19 Incubation Time (h)

the greatest [^{14}C]GA_{20} accumulation followed by 4-Me-IAA (4 and 12 h after radiolabel application). Deseeded pericarps treated with IAA did not stimulate [^{14}C]GA_{20} accumulation above the SPNS control. The accumulation pattern of [^{14}C]GA_{20} in deseeded pericarps treated with GA_3 or GA_3 plus 4-Cl-IAA was different than those treated with auxins. Accumulation of [^{14}C]GA_{20} was low 4 h after radiolabel incubation (similar to the SPNS control), significantly increased by 12 h of incubation, and remained at these levels up to 24 h after radiolabel application (Figure 3-5B).

The difference in the accumulation pattern of [^{14}C]GA_{20} in deseeded pericarps treated with GA_3 or GA_3 plus 4-Cl-IAA and those treated with auxins can be accounted for by the metabolism of [^{14}C]GA_{20} to [^{14}C]GA_{29}. Deseeded pericarps treated with GA_3 or GA_3 plus 4-Cl-IAA accumulated little to no [^{14}C]GA_{29} through 12 h of radiolabel incubation (Figure 3-6B). After 24 h of [^{14}C]GA_{19} incubation, moderate levels of [^{14}C]GA_{29} accumulation are detected in these treatments. In general, in all other treatments [^{14}C]GA_{29} accumulation increased with increasing radiolabel incubation time (Figure 3-6B).

Metabolism of [^{14}C]GA_{29} to putative [^{14}C]GA_{29}-catabolite was detected in pericarp tissue of all treatments except deseeded pericarps treated with GA_3 or GA_3 plus 4-Cl-IAA (Figure 3-7B). Accumulation of putative [^{14}C]GA_{29}-catabolite increased with increasing radiolabel incubation in deseeded pericarps (SPNS) and deseeded pericarps treated with 4-Me-IAA and IAA(Figure 3-7B). Accumulation of putative [^{14}C]GA_{29}-catabolite in deseeded pericarps treated with 4-Cl-IAA was low during the first 12 h of radiolabel incubation, but increased by 24 h of incubation (Figure 3-7). Putative [^{14}C]GA_{29}-catabolite was detected in low amounts in pericarps with seeds (SP) 12 and 24 h after radiolabel application.

Figure 3-6. A) The effect of seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, and IAA and B) the effects of seeds (SP), seed removal (SPNS) and seed removal plus treatment with GA_3 and GA_3 plus 4-Cl-IAA on [^{14}C]GA_{29} accumulation over a 24 h period as described in Figure 3-4. Means ± SE , n=3.


A
4-Cl-IAA
4-Me-IAA
IAA
SPNS
[14C]GA29 (DPM X 10-3)
0
5
10
15
20
25
30
35
40
45
0
4
8
12
16
20
24

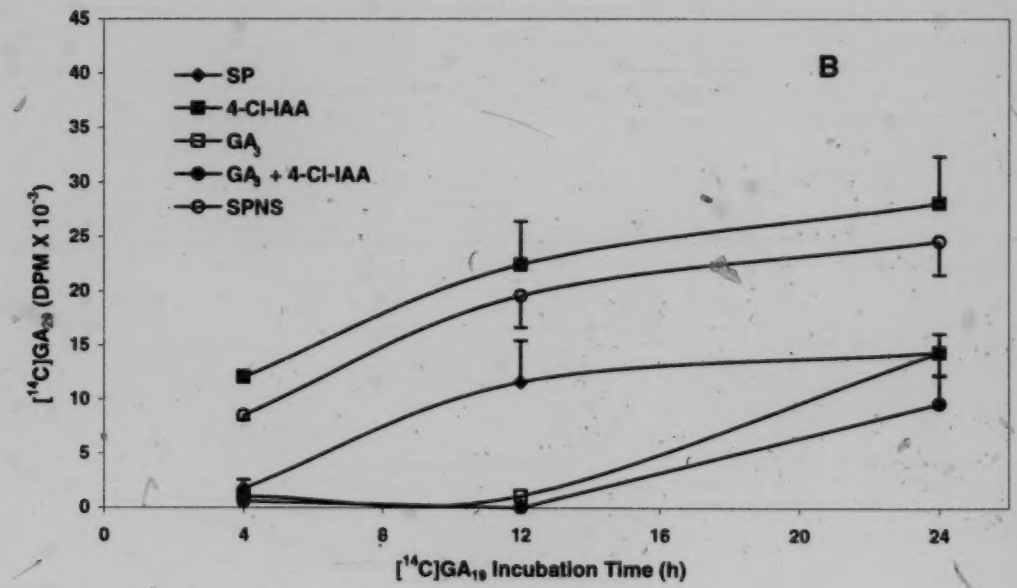
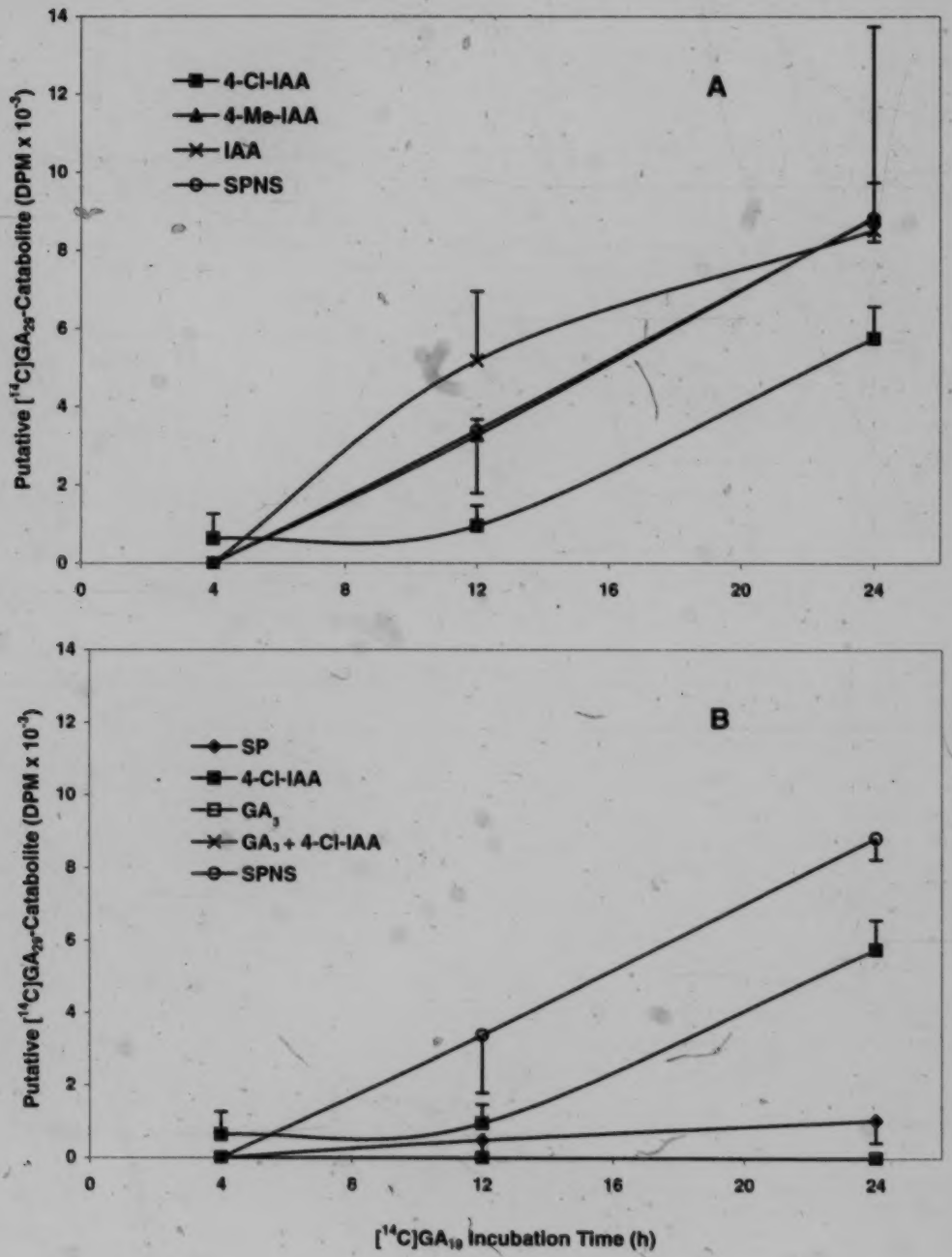

B
SP
4-Cl-IAA
GA3
GA3 + 4-Cl-IAA
SPNS
[14C]GA29 (DPM X 10-3)
0
5
10
15
20
25
30
35
40
45
0
4
8
12
16
20
24
[14C]GA19 Incubation Time (h)

Figure 3-7. A) The effect of seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, and IAA and B) the effects of seeds (SP), seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, GA_3, and GA_3 plus 4-Cl-IAA on putative [^{14}C]GA_{29}-catabolite over a 24 h as described in Figure 3-4. Means ± SE, n = 3.


A
4-Cl-IAA
4-Me-IAA
IAA
SPNS
Putative [14C]GA29-Catabolite (DPM x 10-3)
B
SP
4-Cl-IAA
GA3
GA3 + 4-Cl-IAA
SPNS
Putative [14C]GA29-Catabolite (DPM x 10-3)
[14C]GA19 Incubation Time (h)

The [^{14}C]GA_{20}, [^{14}C]GA_{29} and putative [^{14}C]GA_{29}-catabolite HPLC fractions were pooled, methylated, and re-chromatographed on C_{18} HPLC. [^{14}C]GA_{20} and [^{14}C]GA_{29} methyl-esters eluted as one peak at the same retention time as methylated [^{14}C]GA_{20} and [^{13}C-^{3}H]GA_{29} standards. The putative [^{14}C]GA_{29}-catabolite fraction also eluted as one peak.

The conversion of GA_{20} to GA_{29} (2β-hydroxylation) is recognized as a deactivation step, resulting in loss of GA activity (Hoad et al., 1982). The ratio of GA_{29} to GA_{20} is therefore an indication of the relative amount of GA that has flowed out of the active GA pool at this step of GA biosynthesis. In pericarp with seeds and deseeded pericarp treated with GA_3 or GA_3 plus 4-Cl-IAA, the ratio remained 1 or less for the entire incubation period (Figure 3-8B). The ratio was 1 or less during the first 12 h of incubation in deseeded pericarps treated with 4-Cl-IAA, but increased to 5 after 24 h of radiolabel incubation (Figure 3-8). In deseeded pericarps (SPNS) and deseeded pericarps treated with 4-Me-IAA and IAA, the ratio was above 2 within the first 12 h of incubation and increased 2 [illegible]4 times by 24 h of incubation (Figure 3-8A).

The capacity of [^{14}C]GA_{19} to be metabolized through the GA_{19} to GA_{20} step can be estimated by the sum of the free [^{14}C]GA metabolites produced. The sum of the amount of [^{14}C]GA_{20}, [^{14}C]GA_{29} and putative [^{14}C]GA_{29}-catabolite produced over the 24-h radiolabel incubation period revealed that deseeded pericarps treated with 4-Cl-IAA metabolized [^{14}C]GA_{19} to free [^{14}C]GA metabolites to the greatest extent within the first 12 h of radiolabel incubation, with one exception (similar values obtained by deseeded pericarp treated with 4-Me-IAA 12 h after radiolabel incu[illegible] Figures 3-9A and B). The amount of these free [^{14}C]GA metabolites was similar for p[illegible] with seeds (SP) and without seeds (SPNS), with the free [^{14}C]GA metabolites increasing over the first 24 h of radiolabel incubation (Figure 3-9B). Deseeded pericarps treated with GA_3 plus 4-Cl-IAA and GA_3 alone produced the lowest amounts of free [^{14}C]GA metabolites, w[illegible] the free [^{14}C]GA metabolites increasing over the first 12 h and 24 h of

Figure 3-8. A) The effect of seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, and IAA and B) the effect of seeds (SP), seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, GA_3, and GA_3 + 4-Cl-IAA on the ratio of conversion of [^{14}C]GA_{20} to [^{14}C]GA_{29} in pericarp treated as described in Figure 3-4. Means ± SE, n=3.

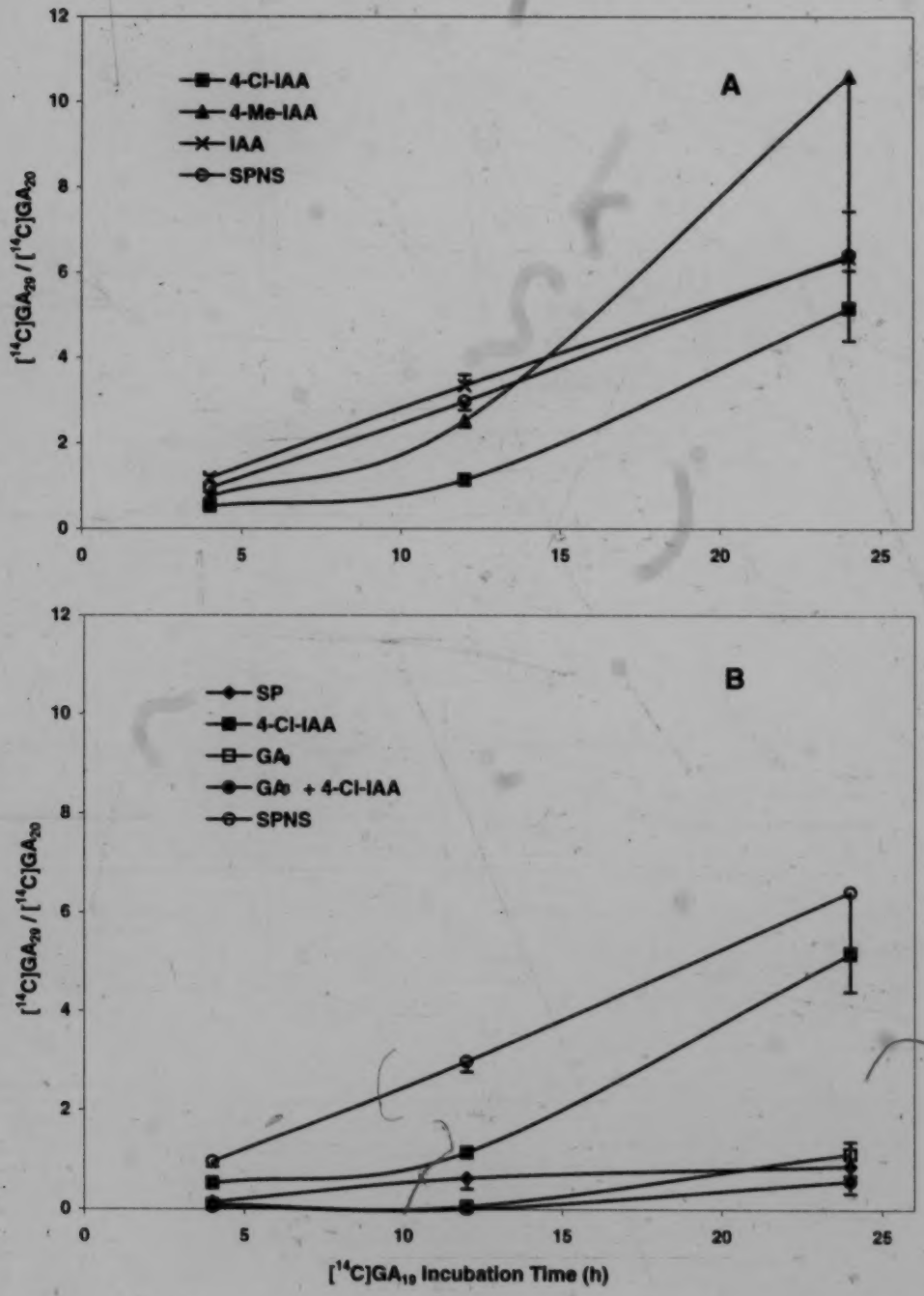
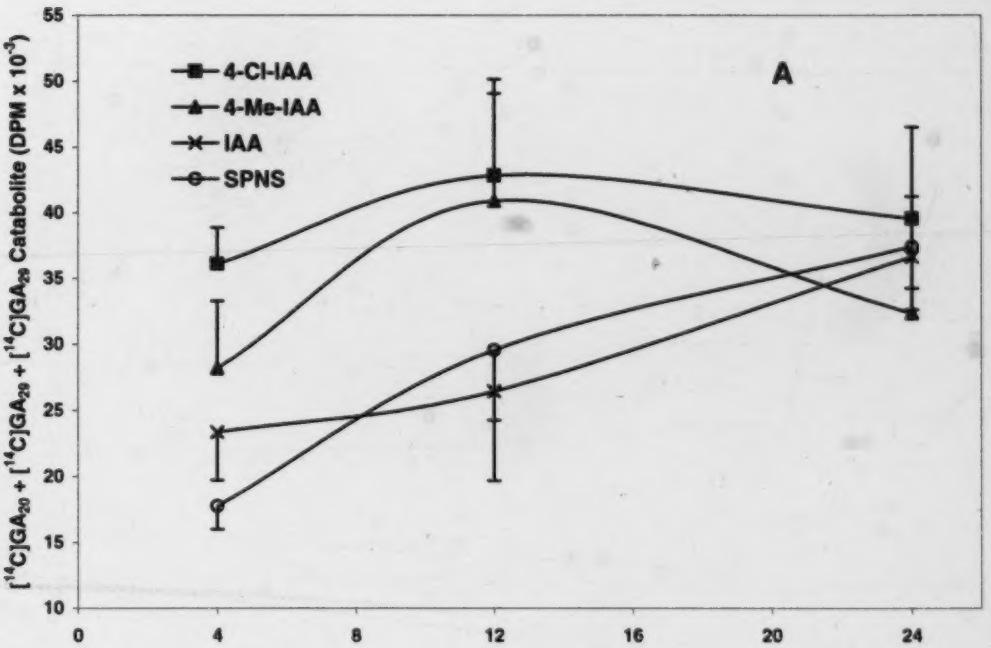
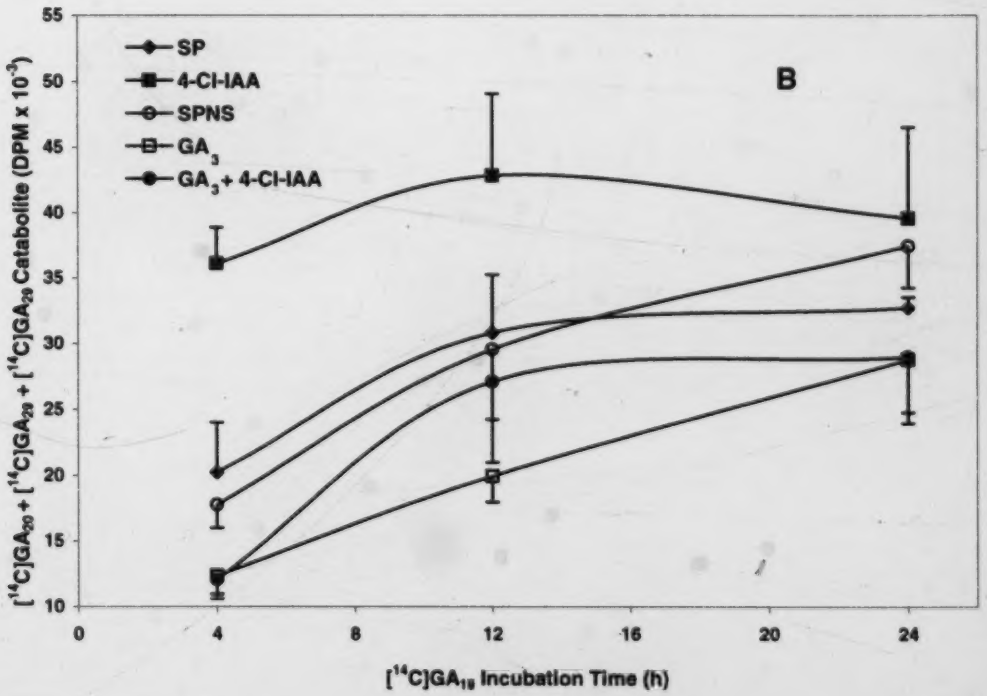

A
4-Cl-IAA
4-Me-IAA
IAA
SPNS
[14C]GA29 / [14C]GA20
B
SP
4-Cl-IAA
GA9
GA9 + 4-Cl-IAA
SPNS
[14C]GA29 / [14C]GA20
[14C]GA19 Incubation Time (h)

Figure 3-9. A)The effect of seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, 4-Me-IAA, and IAA and B) the effect of seeds (SP), seed removal (SPNS) and seed removal plus treatment with 4-Cl-IAA, GA_3, and GA_3 plus 4-Cl-IAA on the added sum of all isolated metabolites ([^{14}C]GA_{20}, [^{14}C]GA_{29} and putative [^{14}C]GA_{29}-catabolite) of pericarp treated as described in Figure 3-4. Means ± SE, n = 3.


4-Cl-IAA
4-Me-IAA
IAA
SPNS
A
[14C]GA20 + [14C]GA29 + [14C]GA29 Catabolite (DPM x 10-3)
55
50
45
40
35
30
25
20
15
10
0
4
8
12
16
20
24


SP
4-Cl-IAA
SPNS
GA3
GA3 + 4-Cl-IAA
B
[14C]GA20 + [14C]GA29 + [14C]GA29 Catabolite (DPM x 10-3)
55
50
45
40
35
30
25
20
15
10
0
4
8
12
16
20
24
[14C]GA19 Incubation Time (h)

radiolabel incubation respectively (Figure 3-9B). Deseeded pericarps treated with IAA produced levels of free [^{14}C]GA metabolites similar to the SPNS control (Figure 3-9B).

The *sln* mutation reduced the levels of [^{14}C]GA_{29} by 3 fold and putative [^{14}C]GA_{29}-catabolite by 7.5 fold in pericarps with seeds (SP; Table 3-1). Concomitantly, the levels of [^{14}C]GA_{20} increased by 1.7 fold in this tissue. In deseeded pericarps (SPNS) the levels of [^{14}C]GA_{20} were low and did not differ between the two genotypes, but levels of [^{14}C]GA_{29} and putative [^{14}C]GA_{29}-catabolite were reduced in the *sln* genotype (by 3 fold and to non-detectable levels, respectively; Table 3-1). In deseeded pericarps treated with 4-Cl-IAA, the *sln* mutation increased the levels of [^{14}C]GA_{20} by 1.8 fold and reduced the levels of [^{14}C]GA_{29} by 3.4 fold and putative [^{14}C]GA_{29}-catabolite to non-detectable levels (Table 3-1).

Putative [^{14}C]GA_1, [^{14}C]GA_3, and [^{14}C]GA_8 were not detected in any treatment or genotype throughout the [^{14}C]GA_{19} 24 h incubation period.

3.4 Discussion

We have shown in the presence of seeds, pericarps were able to metabolize [^{14}C]GA_{19} to [^{14}C]GA_{20}, and upon seed removal, the conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20} was significantly reduced (Figure 3-5). The ratio of [^{14}C]GA_{29} to [^{14}C]GA_{20} (an indication of the relative amount of GA that has flowed out of the active GA pool at this step) remained less than 1 for the entire incubation period in pericarp with seeds. When seeds were removed (SPNS), the ratio was higher than 2 in the first 12 h of incubation and increased 6 fold within 24 h of incubation (Figure 3-8). These data are consistent with previous work by van Huizen et al. (1995). However, the similar capacities of pericarps with and without seeds to metabolize [^{14}C]GA_{19} to free GA metabolites ([^{14}C]GA_{20} + [^{14}C]GA_{29} + putative [^{14}C]GA_{29}-catabolite; Figure 3-9B) varies from data reported by van Huizen et al. (1995) who found that deseeded pericarps had low levels

Table 3-1. Accumulation of [^{14}C]GA_{20}, [^{14}C]GA_{29} and putative [^{14}C]GA_{29} catabolite in WT (SLN) and *sln* mutants after 24 h incubation with [^{14}C]GA_{19}.

	Percent [^{14}C] Metabolite [a, b]		
Treatment	**[^{14}C]GA_{20}**	**[^{14}C]GA_{29}**	**Putative [^{14}C]GA_{29}-Catabolite**
SP (WT; SLN)	7.7% ± 1.1 (12.57 ± 1.83[c])	3.3% ± 1.2 (5.36 ± 1.87)	1.2% ± 0.32 (1.97 ± 0.52)
SPNS (WT; SLN)	1.91% ± 0.14 (3.10 ± 0.31)	0.94% ± 0.23 (1.52 ± 0.36)	1.5% ± 0.59 (2.51 ± 0.96)
4-Cl-IAA (WT; SLN)	5.9% ± 0.76 (11.36 ± 4.23)	14.8% ± 2.5 (24.00 ± 4.05)	4.5% ± 0.43 (7.40 ± 0.70)
SP (*sln*)	13.1% ± 3.9 (16.62 ± 2.21)	1.0% ± 1.0 (1.67 ± 1.67)	0.16% ± 0.16 (0.24 ± 0.24)
SPNS (*sln*)	1.19% ± 1.19 (1.32 ± 1.32)	0.301% ± 0.301 (0.33 ± 0.27)	ND[d]
4-Cl-IAA (*sln*)	10.8% ± 2.5 (15.0 ± 5.75)	4.4% ± 1.3 (6.12 ± 2.71)	ND[d]

[a] Percent calculated as (dpm [^{14}C] metabolite after 24 h incubation) ÷ (dpm[^{14}C] GA_{19} added to tissue) x 100

[b] Amount [^{14}C]GA_{19} added per 3 pods (3pods/extraction): All WT (SLN) 162,525 dpm; SP(*sln*) 155,954 dpm and 4-Cl-IAA (*sln*) 111,054 dpm; SPNS (*sln*) 111,054 dpm

[c] Amount of [^{14}C] metabolite in DPM x 10^{-3} ± SE, n = 3 for SLN (WT) and n = 2 for *sln*.

[d] ND = Not detected

of free [^{14}C]GA metabolites ([^{14}C]GA_{20} + [^{14}C]GA_{29}) compared to pericarp with seeds. One difference between these studies was that the growth of deseeded pericarps was small in the van Huizen et al. (1995) study (4 mm over 48 h) compared to (10 mm over 48 h; Figure 3-3) this study. It is possible that the down-regulation of pericarp GA 20-oxidase conversion of GA_{19} to GA_{20} may lag behind the up-regulation of pericarp 2β-hydroxylation conversion of GA_{20} to GA_{29} after deseeding. If this is the case, pericarps that have a greater capacity for growth after deseeding would accumulate more radiolabeled GA_{29} and GA_{29}-catabolite than slower growing pericarps. Pericarp growth and [^{14}C]GA_{19} metabolism data from the SLN (WT) genotype support this hypothesis. The SLN (WT) pericarps grew only 2 mm after deseeding (Figure 3-3) and deseeded pericarps had low levels of free [^{14}C]GA metabolites ([^{14}C]GA_{20} + [^{14}C]GA_{29} + [^{14}C]GA_{29}-catabolite) compared to pericarps with seeds (Table 3-1).

Application of 4-Cl-IAA to deseeded pericarp stimulated pericarp growth (Figure 3-2), metabolism of [^{14}C]GA_{19} (Figure 3-4), and *in vivo* GA 20-oxidase conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20} (Figure 3-5A) compared to the SPNS control. These data confirm previous work that 4-Cl-IAA can substitute for the seeds in the stimulation of pericarp growth and conversion of GA_{19} to GA_{20} (Reinecke et al., 1995, 1999; van Huizen et al., 1995). 4-Cl-IAA also increased levels of GA20-oxidase mRNA in deseeded pericarp compared to the SPNS control (van Huizen et al., 1997; Chapter 2). Due to different hormone application times for experiments on *in vivo* GA 20-oxidase enzyme conversion of GA_{19} to GA_{20} (van Huizen et al., 1995) and GA 20-oxidase gene expression (van Huizen et al., 1997) in pea pericarp, only a general comparison could be made between these studies. The timing of hormone application is the same between this study and the GA 20-oxidase gene expression study in Chapter 2 allowing a direct comparison of these processes. The highest GA 20-oxidase mRNA levels were observed between 2 and 12 h after 4-Cl-IAA application (8 to 11 fold above the SPNS control, van Huizen et al., 1997; Chapter 2) and

these levels decreased by 24 h after hormone application. The *in vivo* GA 20-oxidase conversion of GA_{19} to GA_{20} was 2.6 fold greater than the SPNS control 4 h after the peak period of GA 20-oxidase expression (16 h after 4-Cl-IAA application; 4 h after [^{14}C]GA_{19} application), and decreased with increasing time after hormone application (Figure 3-5). This comparison supports the data of van Huizen et al. (1997) that the effects of 4-Cl-IAA on the synthesis of bioactive GA via GA_{20} may be significantly mediated by the effects on gene expression.

Application of [^{14}C]GA_{19} to 4-Cl-IAA-treated deseeded pericarps of SLN and *sln* genotypes resulted in an increase of [^{14}C]GA_{20} (3 and 9 times that of the SPNS control; Table 3-1) to levels similar to pericarps with seeds (SP; Table 3-1). These data show that the effects of 4-Cl-IAA on pea pericarp GA 20-oxidase conversion of GA_{19} to GA_{20} and growth are not specific to one cultivar of pea and further support the hypothesis that 4-Cl-IAA acts as a seed-derived signal to stimulate GA biosynthesis in the pea pericarp (conversion of GA_{19} to GA_{20}).

Among the auxin treatments, deseeded pericarp growth was the greatest with 4-Cl-IAA application followed by 4-Me-IAA (Figure 3-2). IAA treatment did not stimulate deseeded pericarp growth above the SPNS control (Figure 3-2). These results are in agreement with those of Reinecke et al. (1995, 1999) who investigated the effects of the 4-position of the indole ring on pericarp growth by assaying activities of various 4-substituted IAAs (4-Cl-, 4-Me-, 4-Et-, 4-F-, and H-IAA). It was determined that the size of the 4-substituent dramatically affected biological activity, with 4-Cl-IAA being most effective in stimulating pericarp growth. van Huizen et al. (1995, 1997) determined the effects of 4-Cl-IAA on *in vivo* GA 20-oxidase conversion of GA_{19} to GA_{20} and GA 20-oxidase mRNA expression, but, it was not known whether these effects were a direct result of bioactive auxin or non-specific effects attributable to auxin-type molecules. As previously mentioned, the timing of hormone applications in this study (enzyme activity) is the same as the GA 20-oxidase gene expression study in Chapter 2 allowing a direct comparison between these processes. GA 20-oxidase mRNA levels in deseeded pericarps treated with 4-Cl-

IAA were the highest (11 times higher than the SPNS control), followed by 4-Me-IAA (5.5 times higher than the SPNS control) within the first 4 h of hormone application and the transcript levels remained elevated above the SPNS control in both treatments during the 24-h experimental period (Chapter 2). Application of IAA to deseeded pericarps resulted in no increase in transcript levels compared to the SPNS control (Chapter 2). The *in vivo* GA 20-oxidase conversion of GA_{19} to GA_{20} (accumulation of [^{14}C]GA_{20}) 4 h after the peak period of GA 20-oxidase mRNA expression (16 h after 4-Cl-IAA application; 4 h after [^{14}C]GA_{19} application) was 2.6 and 1.8 fold greater than the SPNS control for 4-Cl-IAA- and 4-Me-IAA-treated deseeded pericarps, respectively, and decreased with increasing time after hormone application (Figure 3-5A). Application of IAA to deseeded pericarps did not stimulate *in vivo* GA 20-oxidase conversion of GA_{19} to GA_{20} above the SPNS control (Figure 3-5A). These data show that the stimulation of pericarp growth, conversion of GA_{19} to GA_{20}, and GA 20-oxidase mRNA expression are specific to the biologically active auxins in the pea split-pericarp assay.

The conversion of [^{14}C]GA_{20} to [^{14}C]GA_{29} (2β–hydroxylation) is considered a deactivation step, resulting in loss of GA activity (Hoad et al., 1982). In general, the ratio of [^{14}C]GA_{20} to [^{14}C]GA_{29} increased with increasing radiolabel incubation time in deseeded pericarps and deseeded pericarps treated with auxins (with one exception, the ratio of [^{14}C]GA_{20} to [^{14}C]GA_{29} in 4-Cl-IAA-treated deseeded pericarps only increased after 24 h of radiolabel incubation; Figure 3-8) but remained at 1 or less throughout the incubation period in pericarp with seeds (SP). These data suggest that the 2β-hydroxylation of GA_{20} to GA_{29} in the pericarp may be repressed by the seeds as proposed by van Huizen et al. (1995).

In previous work, Ozga et al. (1992) found that GA_3 applied to deseeded pericarps stimulated growth, but conversion of [^{14}C]GA_{12} to putative [^{14}C]GA_{20} was not detected after a 24 h incubation period. These data coincide with data by van Huizen et al. (1997) that showed GA 20-oxidase transcript levels to decrease within 2 h of GA_3 application. However, in this study

application of GA_3 to deseeded pericarps stimulated pericarp growth (Figure 3-2) and accumulation of $[^{14}C]GA_{20}$ after 12 h of $[^{14}C]GA_{19}$ incubation compared to the SPNS control (Figure 3-5). Since GA_{12} is 3 metabolic steps before GA_{19} in the GA biosynthesis pathway, less $[^{14}C]GA_{19}$ would be available for conversion to $[^{14}C]GA_{20}$ by the pericarp when using $[^{14}C]GA_{12}$ as the radiolabeled substrate versus $[^{14}C]GA_{19}$. With the lower amount of $[^{14}C]GA_{19}$ available for conversion to $[^{14}C]GA_{20}$ by the pericarp in the studies using $[^{14}C]GA_{12}$, the metabolic profile of the $[^{14}C]GAs$ in deseeded pericarp treated with GA_3 would more likely reflect the reduction of GA 20-oxidase activity (little to no $[^{14}C]GA_{20}$ accumulation) and less likely reflect the accumulation of $[^{14}C]GA_{20}$ later in the incubation period (after 12 h of radiolabel incubation) due to the reduction in conversion of GA_{20} to GA_{29} as seen in this study.

Thomas et al. (1999) reported GA_3-induced up-regulation of 2β-hydroxylase gene expression (conversion of GA_{20} to GA_{29}) and down-regulation of GA 20-oxidase gene expression in immature flower buds of the GA-deficient *ga1-2* mutant of *Arabidopsis*. These data support the hypothesis that GA_3 controls the levels of bioactive GAs (at these two points in the metabolic pathway) at the transcription level. However, in the present study GA_3 down-regulated both GA 20-oxidase activity (conversion of $[^{14}C]GA_{19}$ to $[^{14}C]GA_{20}$) and 2β-hydroxylase activity (conversion of $[^{14}C]GA_{20}$ to $[^{14}C]GA_{29}$) in pea pericarp. It appears that the regulation of the 2β-hydroxylase at the transcriptional and/or posttranscriptional levels in pea pericarp differs from that found in immature flower buds of *Arabidopsis* (*ga1-2*).

When GA_3 and 4-Cl-IAA were applied simultaneously to deseeded pericarps, the average level of $[^{14}C]GA_{20}$, (although not significantly different due to high variability among replications) was higher than in deseeded pericarps treated with GA_3 alone after 12 h of $[^{14}C]GA_{19}$ incubation (Figure 3-5B). This apparent interaction of 4-Cl-IAA and GA_3 in the regulation of GA 20-oxidase conversion of GA_{19} to GA_{20} coincides with that found at the mRNA

level by van Huizen et al. (1997) where the application of 4-Cl-IAA to deseeded pericarps delayed the down-regulation of GA 20-oxidase mRNA levels by GA_3.

Similar to results reported by van Huizen et al. (1995), metabolism of $[^{14}C]GA_{19}$ to $[^{14}C]GA_1$ was not observed in pea pericarps during the 24-h $[^{14}C]GA_{19}$ incubation period with any treatments. Metabolism of $[^{14}C]GA_{19}$ in pericarps of the *sln* genotype (have elongated stems because of a reduced capacity to convert GA_{20} to GA_{29} resulting in elevated levels of GA_1; Reid et al., 1992) was investigated to determine if $[^{14}C]GA_1$ would be detectable in pericarp tissue that had reduced 2β-hydroxylase activity. Neither the SLN nor *sln* genotypes contained any detectable $[^{14}C]GA_1$. A possible explanation for the lack of detection of GA_1 in our system may be that an incubation period longer than 24 h is required. Maki and Brenner (1991) reported the conversion of $[^{2}H]GA_{53}$ to $[^{2}H]GA_1$ after a 48 h incubation period in pea pericarp with seeds. Alternatively, any $[^{14}C]GA_1$ produced by the pea pericarp may be used immediately upon synthesis and therefore its level may be well below the detectable limits of the radiochemical detector. Also, it is possible that after $[^{14}C]GA_{20}$ is synthesized in the pericarp it is transported to another tissue (seed or vegetative tissue) and converted to $[^{14}C]GA_1$ then transported back into the pericarp. MacKenzie-Hose et al. (1998) found radiolabeled GA_1 in the pericarp after $[^{13}C,^{3}H]GA_{20}$ was applied to nearby leaves in the genotype *lh-2* (a mutation in young pea seeds inhibiting the conversion of *ent*-kaurene to CDP) and suggest that GA_1 could be imported from vegetative tissue. However, these plants were detopped prior to radiolabel application therefore altering (increasing) sink strength of the fruit. GA_1 does not appear to be imported from seeds, since mutants of *ls-1*, and *le-3* did not affect seed GA_1 content, but did substantially reduce GA_1 content in pods (MacKenzie-Hose et al., 1998). The inability to detect radiolabeled GA_1 in the pericarp after application of radiolabeled GA precursors to the pericarp tissue remains a topic for further research.

We have shown that [^{14}C]GA_{19} is converted to [^{14}C]GA_{20} by the pea pericarp when seeds are present and that this conversion is inhibited when seeds are removed. Application of 4-Cl-IAA to deseeded pericarp stimulated pericarp growth and conversion of [^{14}C]GA_{19} to [^{14}C]GA_{20}. These results confirm our previous growth (Reinecke et al., 1995) and metabolism studies using [^{14}C]GA_{12} (Ozga et al., 1992) and [^{14}C]GA_{19} (van Huizen et al., 1995) as metabolic substrates, and show that seeds and 4-Cl-IAA regulate growth and GA metabolism in pea pericarp. Our data also confirm previous pericarp growth studies by Reinecke et al. (1995, 1999) and show that the stimulation of GA 20-oxidase activity is specific to the biologically active auxins in the pea split-pericarp assay system. We have also provided evidence that suggests that GA_3 is a negative regulator of 2β-hydroxylase conversion of GA_{20} to GA_{29} and further metabolism of the 2β-hydroxylated GAs. Seed transmittable factors, such as 4-Cl-IAA, may be responsible for stimulation of GA biosynthesis in the pericarp. The sink strength of the seeds may also be involved in maintaining pericarp GA biosynthesis. Using the pea split-pericarp assay system will help further elucidate the interaction of auxins and GA in pea fruit development.

3.5 Literature Cited

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Chapter 4

Summary and Conclusions

The objective of this research was to test the working hypothesis that endogenous auxin acts as a seed-derived signal to coordinate growth of the surrounding fruit tissue (pericarp). The specific objectives of this study were to:

1) to use the 4-substituted auxins (which were previously determined to possess a range of biological activities in the pea split-pericarp assay system; Reinecke et al., 1999) as molecular tools to determine the specificity of auxin regulation of GA 20-oxidase expression and enzyme activity in pea pericarp.
2) to determine the dose-response relationship of 4-Cl-IAA on GA 20-oxidase expression in pea pericarp.

The results of this research have established that regulation of GA 20-oxidase expression by auxins is specific to the biologically active auxins in the split-pericarp assay system. Northern blot analysis of GA 20-oxidase mRNA levels over time (Figures 2-7, 2-9) have revealed that 4-Cl-IAA was better able to stimulate GA 20-oxidase mRNA expression levels than other synthetic and natural auxins used in the study (4-Me-IAA, 4-Et-IAA, 4-F-IAA, and IAA). These findings provide answers to questions previously asked, whether the effect of GA 20-oxidase mRNA levels was linked to auxin-induced growth or a non-specific effect attributable to auxin-type molecules (van Huizen et al., 1997). Growth of pea pericarp as well as mRNA levels of GA 20-oxidase reveal that regulation of GA 20-oxidase expression is specific to the biologically active auxins. The ability of the 4-substituted auxins to increase the levels of GA 20-oxidase mRNA was associated with their ability to stimulate pericarp growth (Figures 2-2, 2-7 and 2-9).

The metabolic profiles of the 4-substituted auxins were also used to further understand the regulation of GA 20-oxidase through enzyme activity in pea pericarp. Metabolic profiles of several auxins tested revealed [^{14}C]GA_{19} was metabolized most efficiently with the application of 4-Cl-IAA to deseeded pea pericarp (Figure 3-4) compared to other auxins. Increased levels of [^{14}C] labeled metabolites further down the GA biosynthesis pathway i.e. [^{14}C]GA_{20} coincided with growth studies and GA 20-oxidase mRNA expression levels previously carried out. These results suggest that 4-Cl-IAA is an important molecule in stimulating or maintaining GA biosynthesis. It would be of interest however, to determine whether the auxin growth responses, GA 20-oxidase expression and metabolism profiles are due to the differential effects of the 4-substituted auxins on ethylene biosynthesis and/or ethylene action. Through the use of ethylene action inhibitors and ethylene biosynthesis inhibitors, we could determine whether the auxin specific response in pericarp is linked to ethylene action or inhibition. It would also be of interest, to test whether the rate of pericarp growth affects the metabolism of [^{14}C]GA_{19} in SP and SPNS, since data we have collected for this thesis suggest a possible link between rate of pericarp growth and GA_{19} metabolism (Chapter 3). Pericarp would be split or split and deseeded at 2 DAA and radiolabel application to pericarp would be applied at either 2, 4, 8 or 12 h after deseeding to obtain metabolic profiles of pericarp at different rates of growth.

Our studies also show that bioactive GAs (GA_3) down-regulate conversion of GA_{19} to GA_{20} and GA_{20} to GA_{29} (as compared to the up-regulated mRNA of GA 20-oxidase and 2β-hydroxylases in *Arabidopsis ga1-2*; Thomas et al., 1999). It would be of interest to determine whether the mRNA transcripts of 2β-hydroxylase coincide with the down regulation of the conversion of GA_{20} to GA_{29} in pea pericarp. For this to be performed, a *Pisum sativum* cDNA sequence corresponding to the 2β-hydroxylase gene is required in order to probe for the message after application of bioactive GA to the pericarp.

In our metabolism studies, the lack of detection of [^{14}C]GA_1 might be a direction for further study and interest. Several explanations have presented themselves for why GA_1 has not been detected, such as a short incubation period of radiolabel, GA_1 levels are below the detectable limit of the radiochemical detector and perhaps GA_1 is synthesized from other vegetative tissue and imported into the pod (MacKenzie-Hose et al., 1998). Future research could pursue whether the source of GA_1 is synthesized from other tissue, and subsequently imported to the pod. It would therefore be of interest to see if application of [^{14}C]GA_{20} to pods will result in the detection of [^{14}C]GA_{20} in other areas of the plant, thus investigating the possibility that GA_{20} may be transported out of the pod for possible synthesis of [^{14}C]GA_1 in other sites and transported back to the pod.

The relationship of 4-Cl-IAA on GA 20-oxidase expression in pea pericarp was found to be dose-dependent. Increased concentration of 4-Cl-IAA up to 100 μM significantly increased the GA 20-oxidase mRNA levels above all other treatments but did not display significantly greater pericarp elongation over the 50 μM 4-Cl-IAA treatment (Figure 2-6). Reinecke et al. (1995) demonstrated that application of 4-Cl-IAA immediately after deseeding resulted in significant growth differences at concentrations of 50 and 100 μM. It would be of interest to determine whether GA 20-oxidase mRNA levels differ when 50 and 100 μM 4-Cl-IAA are applied immediately or 12 h after seed removal.

In conclusion, 4-Cl-IAA is an important factor in the coordination of growth in pea pericarp. We have provided molecular and metabolic studies that suggests GA 20-oxidase regulation is specific to the biologically active auxins, and that GA 20-oxidase expression is 4-Cl-IAA dose dependent, and [^{14}C]GA_{19} metabolism is stimulated by 4-Cl-IAA and regulated by bioactive GA. The results of this research suggest that coordination of pericarp growth in surrounding tissue involves the interaction of several hormones, GAs and auxins. Further research investigating the effect of growth rate, ethylene action, and physiological effects on

auxin stimulated GA biosynthesis are required to fully understand the true nature of the interaction between auxins, GAs and fruit development.

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Van Huizen R, Ozga JA, Reinecke DM (1997) Seed and hormonal regulation of gibberellin 20-oxidase expression in pea pericarp. Plant Physiol **115**: 1-6

Appendix

A. Autoradiogram Quantitation

In order to quantitate the mRNA of GA 20-oxidase signals from the northern autoradiograms, the autoradiograms showing the strongest GA 20-oxidase bands with minimal background were scanned using an imaging densitometer. Several autoradiograms were produced to obtain an optimal autoradiogram for quantitation, by increasing or decreasing the time of membrane exposure to the x-ray film. An example of various exposure times for northerns of two treatments (SPNS and IAA) probed for Actin are shown in Figure A1 and quantitation of the Actin mRNA signals are presented in Figures A2 and A3. Quantitation of autoradiograms from two of the three exposure periods (1 day and 2 days) resulted in similar signal values and trends and are representative of blots used for quantitation throughout this thesis. However, the exposure for 3 days resulted in overexposure of autoradiograms and these autoradiograms were not used for quantitation (Figures A1, A2 and A3).

B. Effects of Auxin-Stimulated ethylene production on GA 20-oxidase expression

To obtain preliminary data on the effects of auxin-stimulated ethylene production on GA 20-oxidase mRNA levels, a preliminary experiment was carried out using silver thiosulfate (STS), an ethylene action inhibitor. Pericarp were split down the dorsal suture and deseeded at 2 DAA (between 15 and 20 mm length). STS and IAA were applied as described in Table B1. After treatment, the pods were harvested 16 and 36 hours after initial deseeding. Treated pericarps were harvested onto liquid nitrogen and stored at -70°C until RNA extraction.

Figure A1: One, two and three day x-ray exposure times of a time course of actin mRNA accumulation in deseeded pea pericarp treated with IAA (50 μM) or 0.1% (v/v) Tween 80 (SPNS). Two DAA pericarps were split and deseeded and 30 μL of IAA or Tween 80 was applied 12 h after deseeding, rRNA banding is shown prior to membrane transfer (visualized by staining with EtBR).

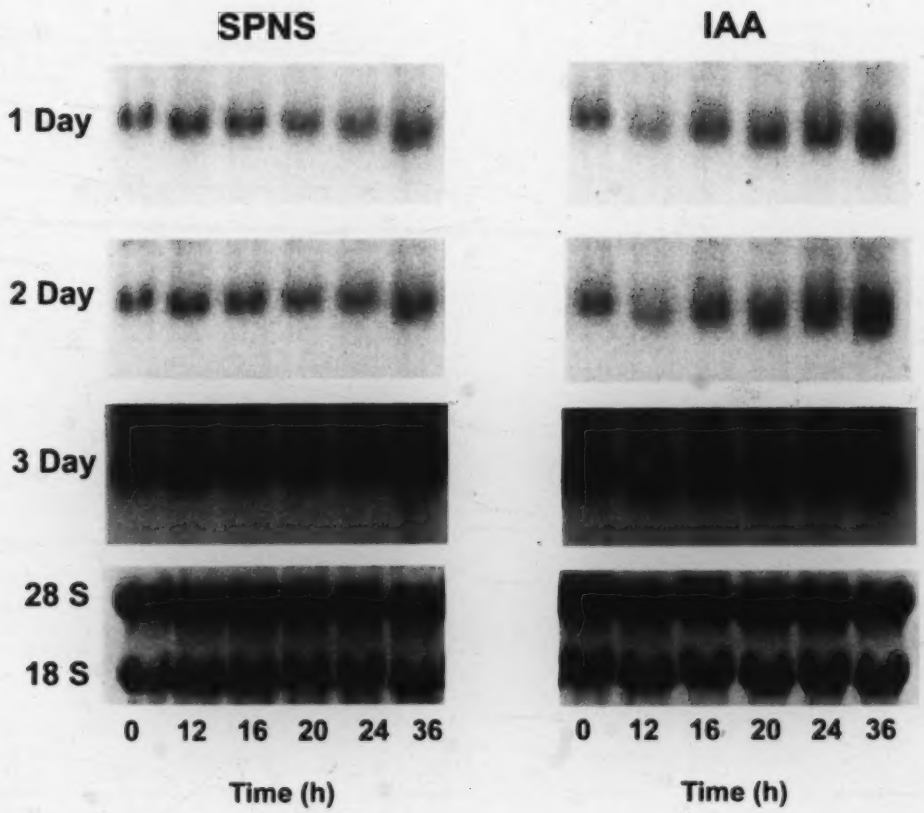
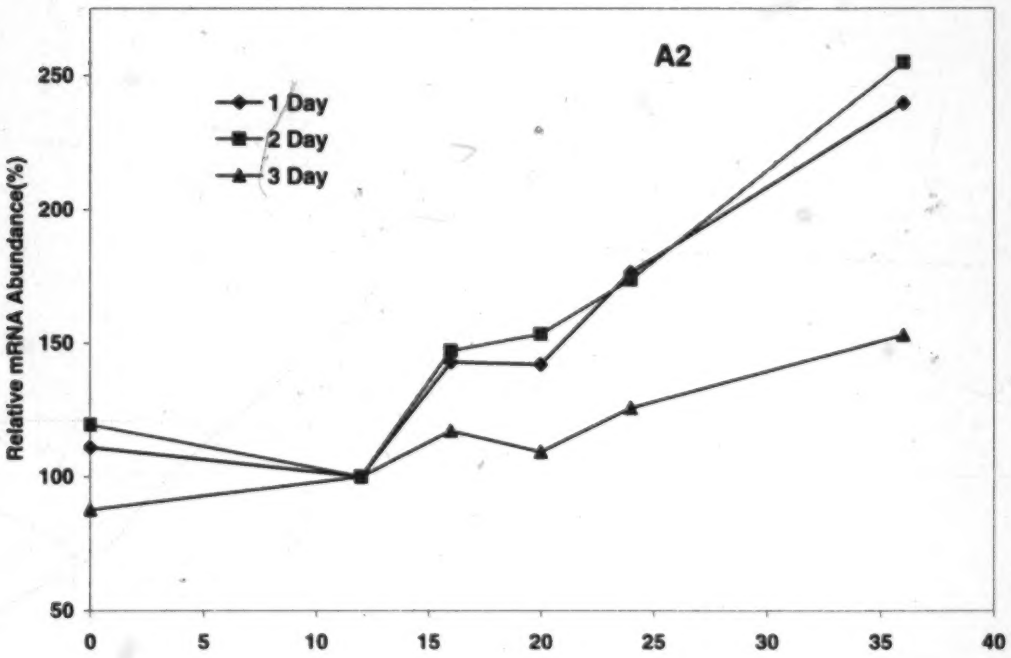

SPNS
IAA
1 Day
2 Day
3 Day
28 S
18 S
0 12 16 20 24 36
Time (h)
0 12 16 20 24 36
Time (h)

Figure A2, A3: Relative mRNA abundance of actin transcripts from deseeded pericarp treated with IAA (A2) or 0.1% (v/v) Tween 80 (SPNS: A3). Each membrane was exposed to X-ray film for 1, 2 or 3 days. Autoradiograms were scanned with an imaging densitometer and values were normalized to the value for pericarps at the time of hormone application (12 h after deseeding).


A2
1 Day
2 Day
3 Day
Relative mRNA Abundance(%)
250
200
150
100
50
0
5
10
15
20
25
30
35
40

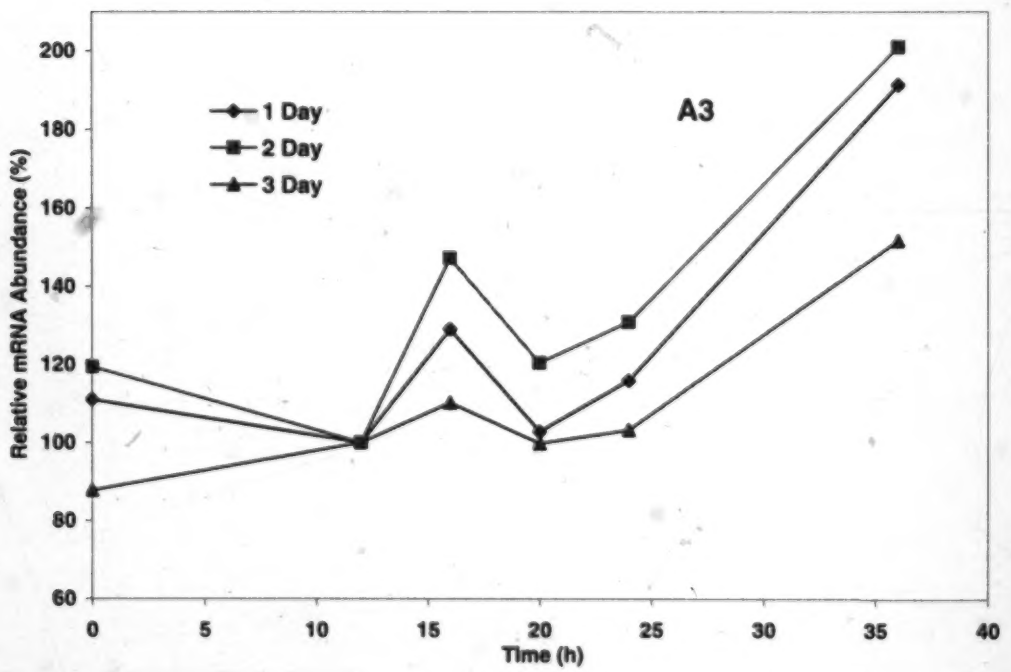

A3
1 Day
2 Day
3 Day
Relative mRNA Abundance (%)
200
180
160
140
120
100
80
60
0
5
10
15
20
25
30
35
40
Time (h)

Table B1. Time Sequence of STS and IAA application to deseeded pericarp

	Time after splitting and deseeding pericarp	
	0 h	**12 h**
Treatment 1 (IAA)	30 µL; 0.1% (v/v) Tween 80	30 µL; 50 µM IAA
Treatment 2 (STS)	20 µL STS	30 µL;0.1% (v/v) Tween 80
Treatment 3 (STS-IAA)	20 µL STS	30 µL; 50 µM IAA
Treatment 4 (SPNS)	---------	30 µL; 0.1% (v/v) Tween 80

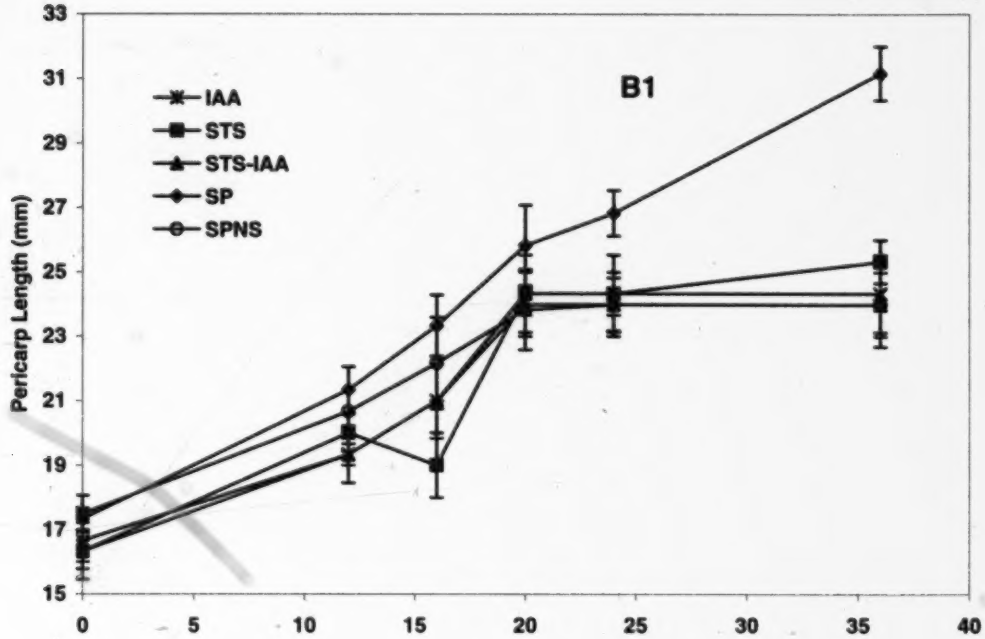
Growth of pericarps with seeds was significantly greater than all other treatments 24 and 36 h after deseeding. No significant difference in growth were observed between pericarps treated with STS and/or IAA during the 36 h experimental treatment (Figure B1).

GA 20-oxidase mRNA levels declined in deseeded pericarps treated with IAA 24 h after hormone treatment (Figures B2, B3). The application of an ethylene action inhibitor (STS) to deseeded pericarp prior to IAA treatment reversed the IAA-induced decline in GA 20-oxidase mRNA levels (Figure B2). This reversal of the IAA-induced decline in GA 20-oxidase mRNA levels with STS treatment suggests that ethylene can also regulate GA 20-oxidase steady state mRNA levels.

C. Effects of Auxins on Ubiquitin mRNA Levels

Initial attempts to use ubiquitin (UBQ4; *Arabidopsis thaliana*; Dr. Joseph Walker; University of Wisconsin) as a loading control for northern blots revealed that the ubiquitin mRNA expression patterns were sometimes similar to patterns observed for GA 20-oxidase in

Figure B1, B2: B1) The effect of seeds (SP), seed removal (SPNS), and seed removal plus treatment with IAA (50 μM), STS, and STS plus IAA on pea pericarp elongation and B2) relative GA 20-oxidase mRNA abundance over a 36 h incubation period. Data are means ±SE, n = 6 (pericarp elongation), n = 2 (GA 20-oxidase mRNA abundance).


B1
IAA
STS
STS-IAA
SP
SPNS
Pericarp Length (mm)
33
31
29
27
25
23
21
19
17
15
0
5
10
15
20
25
30
35
40

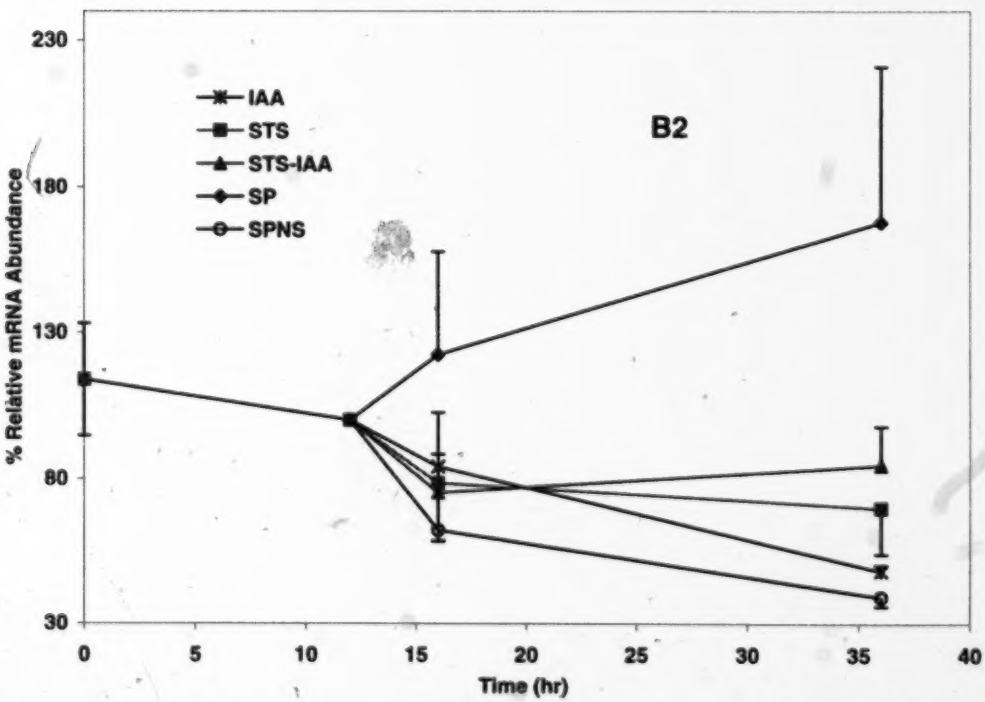
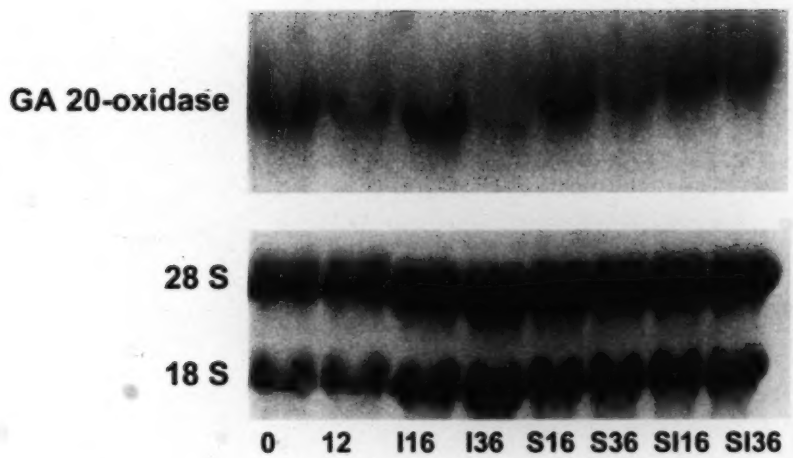

B2
IAA
STS
STS-IAA
SP
SPNS
% Relative mRNA Abundance
230
180
130
80
30
0
5
10
15
20
25
30
35
40
Time (hr)

Figure B3: The effect of IAA (I), STS (S) and STS plus IAA (SI) on GA 20-oxidase mRNA levels in deseeded pericarp. rRNA banding is shown prior to membrane transfer (visualized by staining with EtBr). Pericarps were split and deseeded at t = 0 h and were treated with 20 µL 0.1% (v/v) Tween 80 (IAA treatment) or 20 µL STS (STS and STS plus IAA treatments). At t = 12 h pericarps were treated with 30 µL IAA (50 µM), or 0.1% (v/v) Tween 80 and harvested 16 or 36 h after pericarp deseeding.


GA 20-oxidase
28 S
18 S
0
12
I16
I36
S16
S36
SI16
SI36

deseeded pea pericarp treated with auxins (Figures C1, C2, C3). A 4-Cl-IAA-induced increase in ubiquitin mRNA levels in deseeded pericarp is suggested from these northerns. Also, a more moderate increase in ubiquitin mRNA levels in deseeded pericarp treatment with 4-Me-IAA is indicated. To verify these findings, a dose-response curve of 4-Cl-IAA on ubiquitin mRNA levels could be carried out. These results also indicated that ubiquitin was an inappropriate loading control for the northern blots. As a consequence, a more appropriate control (Actin pAtc4 in pBluescript; *Arabidopsis thaliana*; Dr. Allen Good, University of Alberta) was used.

D. Preliminary Metabolism Experiments

One experiment tested whether reducing the amount of [^{14}C]GA_{19} added to deseeded pericarps would have a significant effect on metabolic profiles and ratios of free labeled-GA products as compared to the standard value of 80,000 dpm/pod used by van Huizen et al. (1997). Three different amounts of radiolabeled substrate were used: 10,000 dpm/pod, 20,000 dpm/pod and 60,000 dpm/pod. Our results (Table D1) indicated that 10,000, 20,000 and 60,000 dpm/pod treatments gave reasonably similar [^{14}C]GA metabolic profiles. Therefore, to obtain more labeled metabolites, 60,000 dpm/pod was used in further experiments.

A second experiment to determine an optimal time course for collection of treated pericarp in order to observe hormonal effects on [^{14}C]GA_{19} metabolism was initiated. In previous experiments by van Huizen et al. (1995) hormones were applied twice, once immediately after seed removal and a second time 20 minutes prior to radiolabel application. In our experimental time course, hormone application was performed only once, 12 h after deseeding and [^{14}C]GA_{19} was applied 12 h after the hormone application. Two time points after radiolabel application were selected to determine GA 20-oxidase enzyme activity, (4 h and 24 h).

Figure C1: Time course of ubiquitin mRNA accumulation in pericarp with seeds (SP), without seeds (SPNS), and without seeds plus 4-Cl-IAA, 4-Me-IAA, 4-Et-IAA, 4-F-IAA and IAA over 36 h. Two DAA pericarps were split or split and deseeded and 30 µL of 50 µM auxin or 0.1% (v/v) Tween 80 (SP and SPNS) was applied 12 h after deseeding.

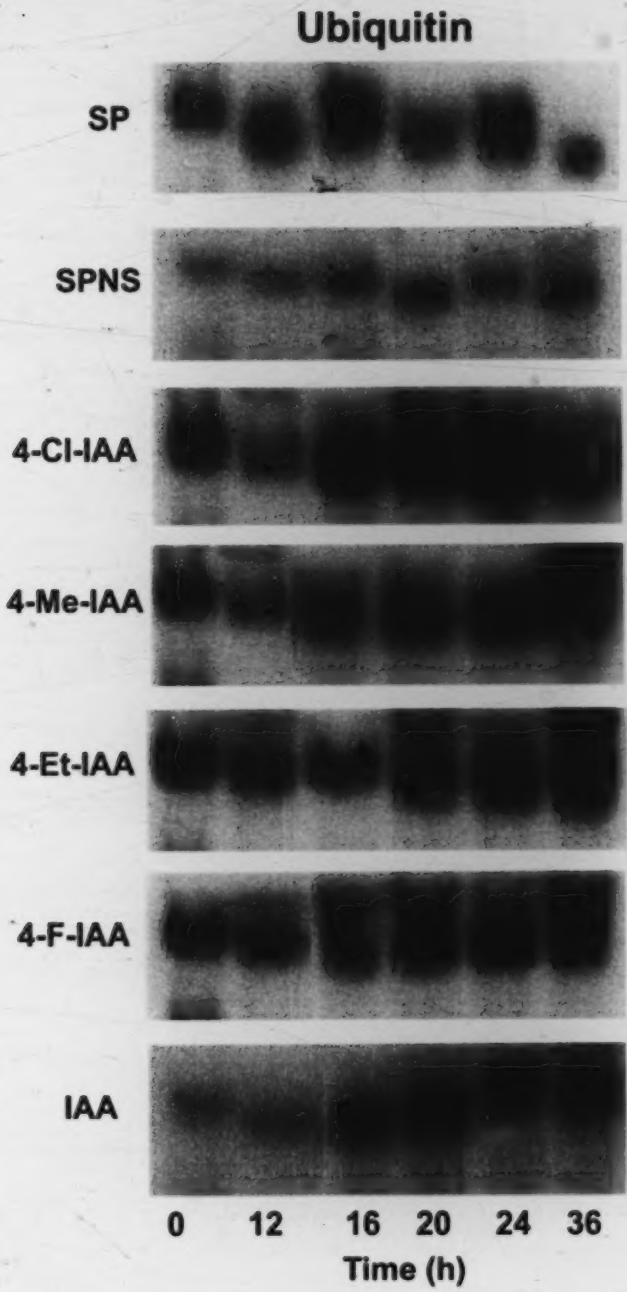
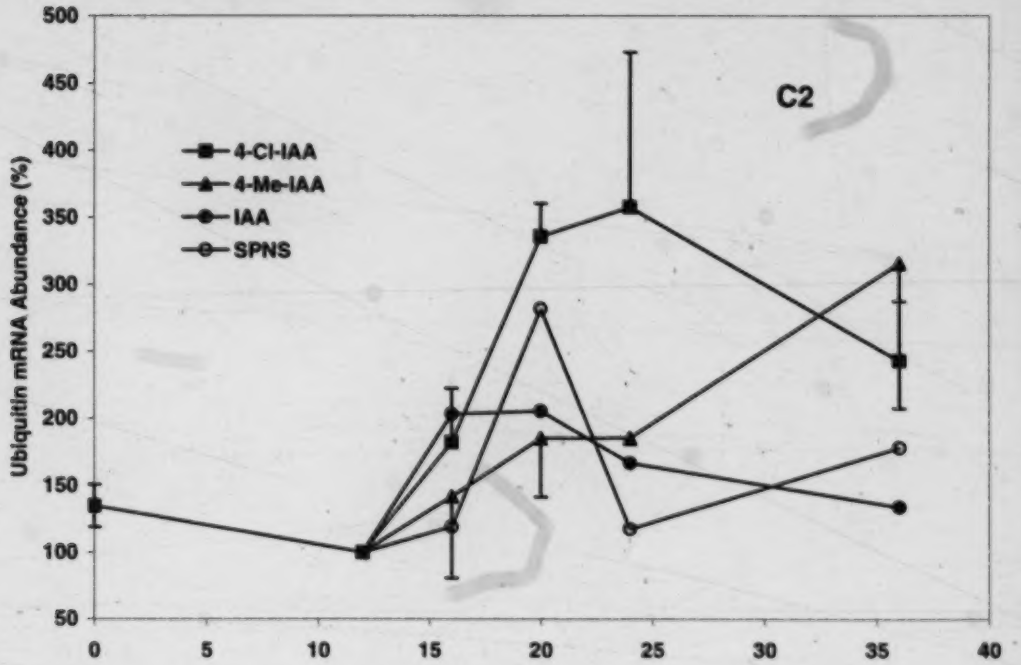

Ubiquitin
SP
SPNS
4-Cl-IAA
4-Me-IAA
4-Et-IAA
4-F-IAA
IAA
0
12
16
20
24
36
Time (h)

Figure C2, C3: Ubiquitin mRNA abundance of deseeded pericarp treated with C2) 4-Cl-IAA, 4-Me-IAA, IAA and 0.1% Tween 80 (SPNS) and C3) 4-Cl-IAA, 4-Et-IAA, 4-F-IAA, and 0.1% (v/v) Tween 80 (SPNS). Two DAA pericarps were split and deseeded and 30 µL of 50 µM auxin or 0.1% (v/v) Tween 80 (SPNS) was applied 12 h after deseeding. Data are means ±SE, n = 3.


C2
4-Cl-IAA
4-Me-IAA
IAA
SPNS
Ubiquitin mRNA Abundance (%)
500
450
400
350
300
250
200
150
100
50
0
5
10
15
20
25
30
35
40

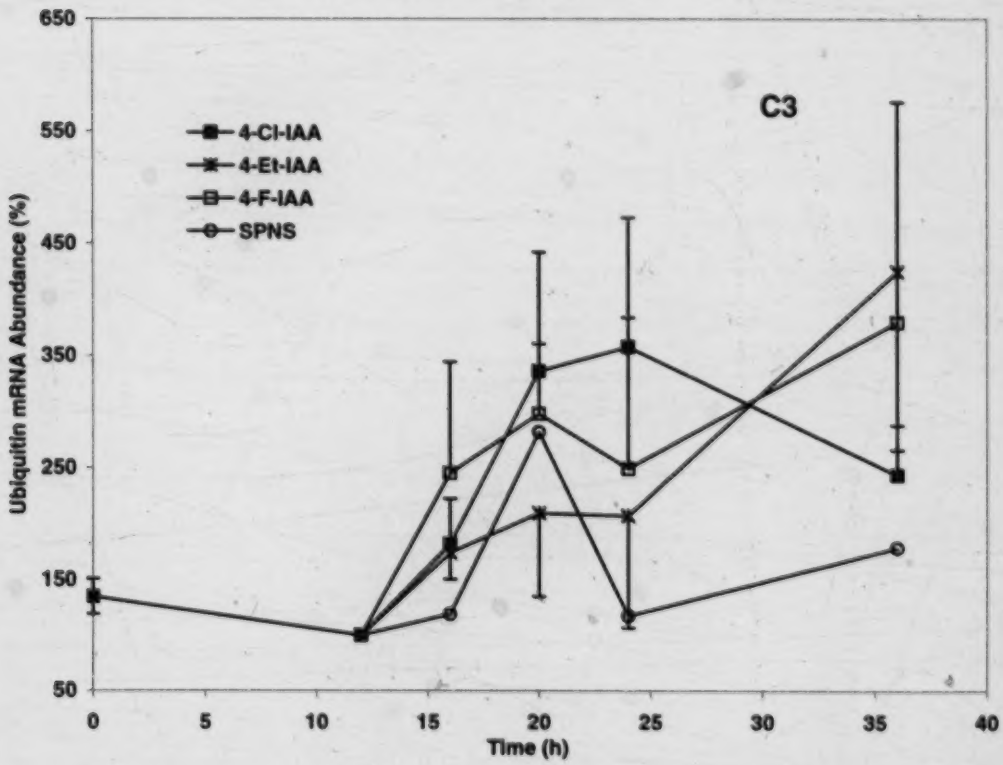

C3
4-Cl-IAA
4-Et-IAA
4-F-IAA
SPNS
Ubiquitin mRNA Abundance (%)
650
550
450
350
250
150
50
0
5
10
15
20
25
30
35
40
Time (h)

Table D1. [^{14}C]GA metabolites produced by deseeded pericarps treated with 4-Cl-IAA (50 μM) after application of 10,000, 20,000, or 60,000dpm of [^{14}C]GA_{19}[a].

[^{14}C]GA_{19} added / pod (3 pods/extraction)	DPM recovered at the HPLC step				
	[^{14}C]GA_{19}	[^{14}C]GA_{20}	[^{14}C]GA_{29}	Putative [^{14}C]GA_{29}-Catabolite	23 min peak
10,000dpm	9796 ± 3033[b] (32%)[c]	2557.5 ± 2557.5 (8.5%)	4427.84 ± 1383.16 (15%)	0	0
20,000dpm	24,737 ± 47 (41%)	12,846.5 ± 35.5 (21%)	8571.5 ± 3120.5 (14%)	0	1209.5± 1209.5 (2%)
60,000dpm	47,005.5 ± 5671.5 (26%)	29,910.5 ± 3710.5(16%)	9724 ± 4839 (5.4%)	2254.5 ± 2254.5(1.3%)	5786.4 ± 516.3 (3.2%)
[d]80,000dpm	---	13,000 ± 100 (5.4%)	17,000 ± 200(7.1%)	---	---

[a] 4-Cl-IAA applied 12 h after deseeding; [^{14}C]GA_{19} applied 24 h after deseeding; pericarp harvested 4 h after [^{14}C]GA_{19} application

[b] dpm ±SE, n = 3.

[c] Percent calculated as (dpm [^{14}C] metabolite after 4 h radiolabel incubation) ÷ (dpm [^{14}C]GA_{19} added to tissue) x 100

[d](2 pods/extraction, from van Huizen et al., 1995)

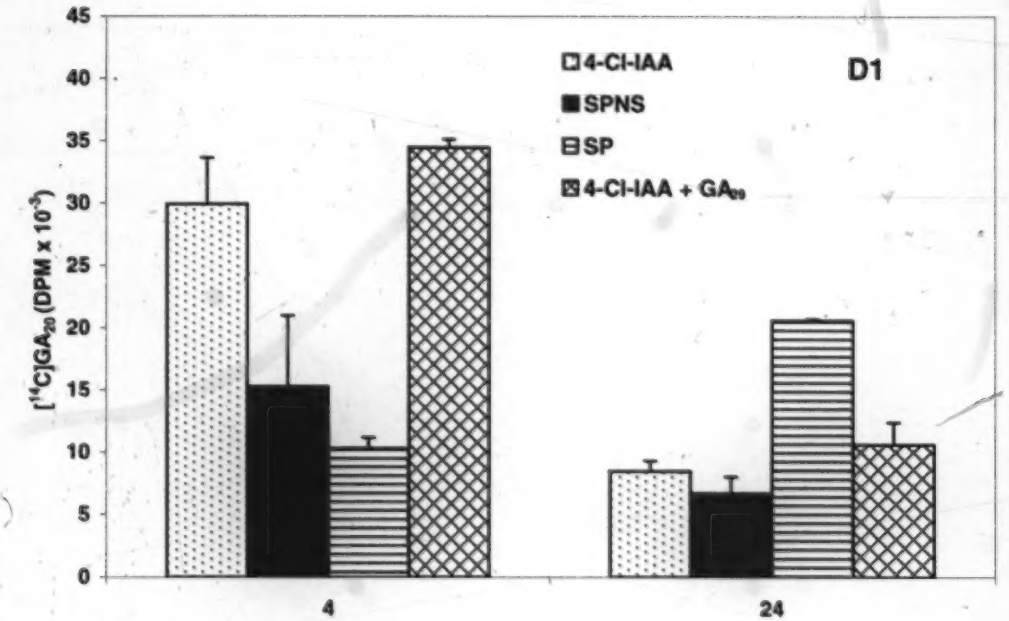
Pericarps from all treatments synthesized [^{14}C]GA_{20} 4 h after hormone treatment (Figure D1). 4-Cl-IAA-treated pericarps synthesized the largest amount of [^{14}C]GA_{20} 4 h after hormone treatment, and by 24 h after treatment these levels declined. [^{14}C]GA_{20} levels increased in pericarp with seeds (SP) over the 24 h incubation period (Figure D1), these results are consistent with data presented by van Huizen et al. (1995). All treatments show a general increase in [^{14}C]GA_{29} accumulation over time (Figure D2).

Treatment of deseeded pericarps with 4-Cl-IAA + GA_{29} was carried out to determine whether blocking [^{14}C]GA_{20} conversion to [^{14}C]GA_{29} by adding exogenous GA_{29} would result in the detection of [^{14}C]GA_1. No [^{14}C]GA_1 metabolite was detected at the HPLC step in these pericarps. However, it should be noted that slightly more [^{14}C]GA_{20} was obtained 4 h after hormone treatment and less [^{14}C]GA_{29} 24 h after hormone treatment in the deseeded pericarps treated with 4-Cl-IAA + GA_{29} (Figures D1 and D2).

Lastly, the application of paclobutrazol (GA biosynthesis inhibitor) to deseeded pea pericarp prior to 4-Cl-IAA application was carried out to determine if a greater amount of [^{14}C]-GA metabolite would be obtained. The application of paclobutrazol should decrease the amount of endogenous GAs present in the system and this may result in a greater amount of free [^{14}C] GAs produced by the pericarp after application of [^{14}C]GA_{19}. In general, the application of paclobutrazol did moderately increase the amount of free [^{14}C] GA metabolites produced by the pericarp (larger amount of [^{14}C]GA_{29} and [^{14}C]GA_{29} catabolite were detected; Table D2).

The final preliminary study was carried out to determine if application of [^{14}C]GA_{20} to the pea pericarp system would result in detection of other GAs further down the GA biosynthesis pathway. Results obtained did not reveal any GAs other than GA_{20}, and GA_{29} (data not shown).

Figure D1, D2: D1) Effects of pericarp with seeds (SP), without seeds (SPNS), and without seeds plus 4-Cl-IAA, and 4-Cl-IAA plus GA_{29} on [^{14}C]GA_{20} levels and D2) levels of [^{14}C]GA_{29} at 4 h and 24 h after [^{14}C]GA_{19} application. Data are means ±SE, n = 2.


D1
4-Cl-IAA
SPNS
SP
4-Cl-IAA + GA29
[14C]GA20 (DPM x 10-3)
45
40
35
30
25
20
15
10
5
0
4
24

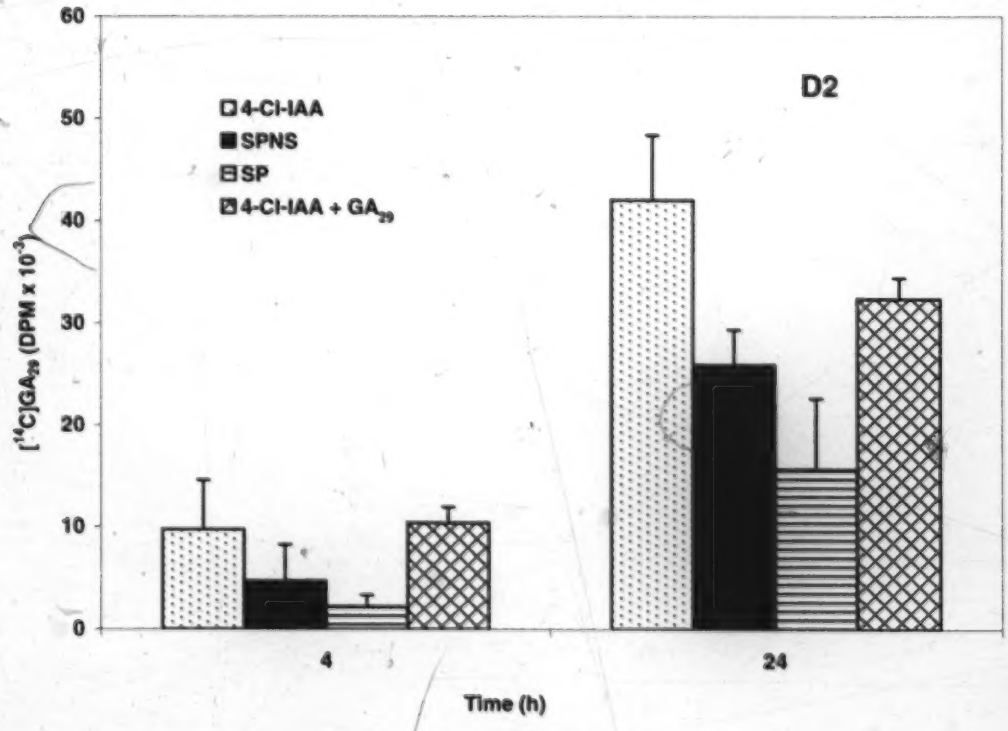

D2
4-Cl-IAA
SPNS
SP
4-Cl-IAA + GA29
[14C]GA29 (DPM x 10-3)
60
50
40
30
20
10
0
4
24
Time (h)

Table D2. [^{14}C]GA metabolites produced by deseeded pericarp treated with 4-Cl-IAA or Paclobutrazol plus 4-Cl-IAA after application of [^{14}C]GA_{19}[a].

Treatment	DPM recovered at HPLC				
	[^{14}C]GA_{19}	[^{14}C]GA_{20}	[^{14}C]GA_{29}	Putative [^{14}C]GA_{29}-Catabolite	23 min peak
4-Cl-IAA	80,440 ± 43,546[b] (45%)[c]	17,198 ± 8815 (9.6%)	12217 ± 2779 (6.8%)	1734 ± 1734 (1.0%)	4372 ± 4372 (2.4%)
Paclobutrazol + 4-Cl-IAA	37,362 ± 4671(21%)	10323 ± 872 (5.7%)	21,285 ± 4328 (12%)	4334 ± 171(2.4%)	4019 ± 1751 (2.2%)

[a] Paclobutrazol added at deseeding (t = 0 h), 2nd hormone application (4-Cl-IAA) applied 23 h after deseeding, [^{14}C]GA_{19} at t = 24 h.

[b] 60,000 dpm/pod, 3 pods/extraction, n = 2.

[c] percent calculated as (dpm [^{14}C] metabolite after 4 h radiolabel incubation) ÷ (dpm [^{14}C]GA_{19} added to tissue) x 100

E. Ent-kaurene synthetase A

Early work was performed to determine if seeds and/or auxin regulate other GA biosynthesis enzymes, specifically *ent*-kaurene synthetase A (KSA). Unfortunately, time constraints did not allow for the full completion of this objective, however, results that were obtained for this objective will be presented here. Briefly, KSA is an enzyme involved in early GA biosynthesis, the conversion of geranyl-geranylpyrophosphate into copalyl diphosphate (CDP). CDP is then converted into *ent*-kaurene, the synthesis of *ent*-kaurene is considered the first committed step in the GA biosynthesis pathway (Sponsel, 1995). It is therefore a key regulatory step in GA biosynthesis and understanding KSA regulation would be an important step in further comprehending hormonal interactions and GA biosynthesis.

The majority of the earlier work focused on the development of a KSA antisense probe, the purpose of which was to carry out RNA protection assays (RPA) to monitor KSA mRNA levels in pea pericarp. Northern blot analysis can not be used in the KSA study as reports by Ait-Ali.et al. (1997) have suggested a very low abundance of KSA mRNA in pea pericarp.

To make the KSA antisense probe, the polymerase chain reaction (PCR) was used to amplify a small fragment of a KSA cDNA clone from pea in a PCR-II vector using two primers, (15F and M13 reverse; clone and primers courtesy Tahar Ait-Ali; Institute of Physical and Chemical Research, Wako-Shi, Japan). Problems were encountered when an extra band was found to be separating closely to the expected 480 bp band of interest. Several PCR attempts and gene cleaning procedures were required in order to isolate enough DNA of the two bands to transform into pCR-Script SK (+) and sequence (Stratagene). Upon sequence analysis it was confirmed that the band of interest was indeed the lower migrating band of ~500 bp. Since the collection and treatment of pod tissue, no further plans have been made to complete this objective.

F. Literature Cited

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